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Polyphenols neuroprotective effect in a Parkinson's disease yeast model: phosphoproteome alterations

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"A lesson without pain is meaningless. For you cannot gain anything without sacrificing something else in return."

Hiromu Arawaka

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Abstract

Parkinson's disease (PD) is a massive chronic progressive disorder characterized by the intracellular depositions known as Lewy Bodies (LBs), mainly composed of alpha-synuclein protein (aSyn). These proteinaceous aggregates are associated with the loss of dopaminergic neurons. aSyn aggregation is thought to be a key event in the PD pathogenesis leading to the formation of LBs.

Thus is extremely important to search and explore therapeutic agents that prevent or reduce aSyn toxicity. The consumption of Polyphenols-rich foods and beverages has been suggested to limit the neurodegeneration associated with a variety of neurological disorders and to prevent or reverse abnormal deteriorations in cognitive performance. These metabolites are consider to exert modulatory actions on cellular systems through direct action on various signalling pathways.

In this study it was analysed the effects mediated by a polyphenol-enriched fraction (PEF) from leaves of *Corema album* in a yeast model of PD expressing aSyn protein. In an effort to obtain new insights into the protective mechanisms of C. album leaf PEFs, as well as the signaling pathways potentially affected by their bioactivity and by aSyn expression, we performed a two-dimensional electrophoresis based quantitative and phosphoproteomic analysis.

It was possible to find several proteins that were either differentially expressed or differentially phosphorylated in response to aSyn expression or due to the incubation of yeast cells with *C. album* leaf PEFs. Also, the results obtained from growth and immunodetection assays suggest that *C. album* PEFs are able to protect yeast cells from the aSyn induced toxicity increasing their viability.

These results imply *C. album* polyphenols from leaves, as potential therapeutic agent directed towards preventing the aSyn induced toxicity. The understanding of the molecular effects of polyphenols will open novel opportunities for the exploration of their molecular effects and for drug development.

Keywords: Parkinson's disease, aSyn, polyphenols, yeast, proteomics.

Resumo

A doença de Parkinson (DP) é uma desordem neurodegenerativa, progressiva e crónica caracterizada por deposições intracelulares denominadas corpos de Lewy (CL), que são maioritariamente compostos pela proteína alfa-sinucleína (aSyn). Estes agregados proteináceos estão associados com a degeneração dos neurónios dopaminérgicos.

Assim é extremamente importante investigar possíveis agentes terapêuticos que sejam capazes de reduzir ou prevenir a toxicidade da aSyn. O consumo de alimentos e bebidas ricos em polifenóis estão associados a uma limitação na neurodegeneração associada a várias desordens neurológicas e a prevenir ou reverter deteriorações na performance cognitiva. Estes metabolitos exercem alterações nos sistemas celulares através do interação com várias vias de sinalização.

Neste estudo foi analisado o efeito de frações enriquecidas em polifenoís (FEPs) de folhas de *C. album* num modelo de PD em levedura com expressão da proteína aSyn. Num esforço para obter novo conhecimento em relação ao mecanismo de proteção dos PEFs da folha de *C. album*, assim como, potencias vias de sinalização afetadas pela sua bioatividade e pela expressão da aSyn, foi realizada uma análise quantitativa e fosfoproteomica através da eletroforese bidimensional.

Foi possível encontrar várias proteínas que são diferencialmente expressas ou fosforiladas em resposta à expressão da aSyn ou devido à incubação das leveduras com PEFs da folha de *C. album*. Além disso, os resultados obtidos a partir dos crescimentos celulares e ensaios de imunodetecção sugerem que os PEFs da folha de *C. album* são capazes de proteger as leveduras a partir da toxicidade induzida pela aSyn e aumentar a viabilidade celular.

Estes resultados implicam os polifenóis de folhas de *C. album*, como potencial agente terapêutico para prevenir a toxicidade induzida pela aSyn. A compreensão dos efeitos moleculares dos polifenóis abrirá novas oportunidades para a exploração dos seus efeitos moleculares e desenvolvimento de agentes terapêuticos.

Palavras-chave: Doença de Parkinson, aSyn, polifenóis, levedura, proteomica.

Resumo alargado

As doenças neurodegenerativas são caracterizadas por morte neuronal excessiva e prematura em regiões focais do cérebro. Entre elas a doença de Parkinson (DP) é uma das mais comuns.

Em termos patológicos a DP é caracterizada por uma perda profunda e progressiva dos neurónios dopaminérgicos, localizados na SNpc do mesencéfalo, região localizada na porção superior do tronco encefálico, originando reduções de dopamina na via nigroestriatal. Esta perda progressiva dos neurónios dopaminérgicos está deve-se à presença de corpos de Lewy (CL), agregados proteicos compostos maioritariamente pela proteína aSyn. aSyn é uma proteína que está presente nos terminais pré-sinápticos onde se encontra associada ao trafego intracelular.

A agregação desta proteína em CL esta associada a vários processos patológicos como disfunção mitocondrial e consequente stress oxidativo, disfunção do sistema da ubiquitina/proteassoma (UPS) e vias de autofagia, e bloqueio da libertação de dopamina nos terminais pré-sinápticos.

Uma vez que a medicação atual apenas atenua sintomas da DP, sem parar ou retardar a degeneração de neurónios dopaminérgicos, existe uma necessidade urgente para a identificação de novos agentes terapêuticos que sejam capazes de contrariar a toxicidade da aSyn. Os polifenóis ou compostos fenólicos são fitoquímicos amplamente distribuídos no reino vegetal. Os polifenóis estão presentes em plantas, frutas e vegetais, incluindo pequenos frutos, azeite e vinho. O consumo de alimentos ricos em polifenóis tem sido associado a efeitos benéficos para a saúde humana. Estudos indicam que existe uma relação entre o consumo de alimentos ricos em polifenóis e uma diminuição da incidência de doenças neurodegenerativas.

Estes compostos estão envolvidos num largo espectro de mecanismos de ação celulares, como é o caso da regulação de vias de sinalização relacionadas com a modulação de genes envolvidos em mecanismos morte sobrevivência e proliferação celular, regulação da função mitocondrial e ativação de moléculas antioxidantes endógenos. Assim, os compostos fenólicos surgem como candidatos a possíveis agentes terapêuticos na DP.

A levedura *Saccharomyces cerevisiae* é um dos modelos biológicos mais versáteis para estudar doenças neurodegenerativas, devido ao facto de mecanismos relacionados com a disfunção mitocondrial e proteossomal e regulação transcricional serem

extremamente bem conservados entre leveduras e humanos, possibilitando assim estudar os mecanismos envolvidos neste tipo de doenças.

Neste estudo o principal objetivo consiste em analisar o efeito de frações enriquecidas de polifenoís (FEPs) de folhas de *Corema album*, uma espécie Portuguesa, no proteoma e fosfoproteoma de um modelo biológico de PD em levedura. Com esta análise, torna-se possível estudar a rede de proteínas do nosso modelo de PD em levedura, bem como a modulação na expressão proteica mediada pelos polifenoís d*e C. album.*

O primeiro passo deste trabalho consistiu na otimização de um modelo de PD em levedura. O modelo e composto por 3 estirpes de S. cerevisiae; aSyn-1 e aSyn-2 ambas com o mesmo genoma *(MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1 GAL1pr-syn WT::TRP1 GAL1pr-syn WT::URA3)* que possuem um plasmídeo integrativo, com 2 cópias do gene *SNCA* (aSyn). A expressão da proteína aSyn é regulada por um promotor da galactose. Por ultimo, a estirpe controlo não apresenta o gene *SNCA* (*can1-100 his3-11 15 leu2-3 112 pRS304::TRP1 pRS306::URA3 ade2-1).*

Numa primeira análise foram realizadas curvas de crescimento para as estirpes aSyn-1, aSyn-2 e controlo em diferentes meios de cultura. Os resultados obtidos indicam que a expressão da proteína aSyn nas estirpes aSyn-1 e aSyn-2 é toxica levando a decréscimo na viabilidade celular das leveduras. Contudo, os resultados mostram que a estirpe aSyn-2 é mais sensível aos efeitos tóxicos de expressão aSyn, portanto, esta estirpe foi selecionada para os estudos posteriores.

A próxima etapa permitiu avaliar o efeito exercido por FEPs obtidas a partir de folhas de *C. album* em leveduras que expressam a proteína aSyn. Assim, usando duas metodologias, curvas de crescimento e "spot assays", as estirpes controlo e aSyn-2 foram incubadas com várias concentrações de FEPs de folhas de *C. album.* Em ambos os métodos foi possível observar que várias concentrações de FEPs de folhas de C. album reduzem a toxicidade induzida pela expressão da aSyn na estirpe aSyn-2, e promovem uma melhoria na viabilidade celular.

Numa fase mais avançada do trabalho, a análise do proteoma total e fosfosproteoma por eletroforese bidimensional revelou que a expressão da aSyn e o tratamento das leveduras com FEPs de folhas de *C. album* promovem alterações na expressão proteica de várias proteínas. A análise do proteoma total permitiu encontrar 22 proteínas que apresentaram variações seu padrão de expressão. Curiosamente foi verificado que a maioria dessas proteínas se encontra "downregulated" devido a expressão da aSyn, mas fascinantemente, o tratamento com PEFs recupera o padrão

normal de expressão dessas proteínas. Estes resultados sugerem que os PEFs de folhas de *C. album* possivelmente estão a modular vias sinalização inerentes à aSyn.

Relativamente à análise do fosfoproteoma das estirpes controlo e aSyn-2, foram detetadas 10 proteínas que apresentaram variações no nível de fosforilação. Curiosamente foi observada uma tendência no nível de fosforilação apresentado por estas proteínas, onde a incubação das leveduras da estirpe aSyn-2 com FEPs reduz o nível de fosforilação comparativamente às leveduras da mesma estirpe que não receberam o tratamento. Foi observado que a expressão da aSyn aumenta a fosforilação na estirpe aSyn-2, relativamente estirpe controlo. A modulação dos níveis de fosforilação das proteínas devido ao tratamento com FEPs de folhas de *C. album* indica que estes compostos provavelmente possuem a capacidade de ativar ou inibir vias de sinalização relacionadas com a fosforilação proteica. Sabendo que a fosforilação da aSyn está implícita na formação dos CL, estes resultados sugerem que os polifenóis provenientes das folhas de *C. album* podem ser promissores agentes terapêuticos na DP.

Observou-se ainda através de ensaios de imunodeteção que leveduras da estirpe aSyn-2 que foram incubadas com FEPs de *C. album* exibem níveis inferiores da proteína aSyn relativamente a leveduras que não tiveram o mesmo tratamento com FEPs. Tendo em conta vários estudos indicando que os polifenóis são agentes capazes de modular os sistemas de autofagia celular, estes resultados sugerem que a proteção mediada pelos FEPs da folha da *C. album* pode estar relacionada com a degradação da aSyn via autofagia.

Apesar dos resultados promissores obtidos neste trabalho, estudos envolvendo a seleção de subproteomas serão necessários para uma melhor compressão dos mecanismos subjacentes aos efeitos neuroprotetores mediados pelos polifenóis das folhas de *C. album*.

Palavras-chave: Doença de Parkinson, levedura, polifenóis, aSyn, Frações enriquecidas de polifenóis

General index

Index of figures

Index of tables

Abbreviations

2-DGE - Two-dimensional gel electrophoresis

A30P - Alanine to proline substitution at aSyn amino acid residue 30

A53T - Alanine to threonine substitution at aSyn amino acid residue 53

AD - Alzheimer's disease

aSyn - Alpha-synuclein

ATP - Adenosine tri-phosphate

CBB - Commassie Brilliant Blue

CL - corpos de Lewy

CHAPS - 3[(3-Cholamidoproply)dimethylammonio]-propanesulfonic acid

CNS - Central Nervous System

CSM - Complete supplement mixture

DA - Dopamine

DSB lab - Disease and Stress Biology lab

FEPs - Frações enriquecidas de polifenoís

DT - Doubling time

DTT - Dithiothreitol

E46K - Glutamic acid to lysine substitution at aSyn residue 46

ER - Endoplasmatic reticulum

GAE - Gallic acid equivalents

IEF - Isoelectric Focusing

IMM - Instituto de Medicina Molecular

ITQB - Instituto de Tecnologia Química e Biológica

LB - Lewy bodies

MS - Mass Spectrometry

MW - Molecular Wieght

NAC - Non-Aβ-component

OD_{600nm} - Optical Density at 600 nm

PBS - Phosphate Buffer Saline

PD - Parkinson's disease

PEF - Polyphenols enriched fractions

pI - Isoelectric Point

PTM's - Postranslational Modifications

ROS - Reactive oxygen species

SDS-PAGE - Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SPE - Solid phase extraction

UPS - Ubiquitin-proteasome system

WT - Wild type

YPD - Yeast extract, peptone and dextrose

SNpc - "*Substancia nigra pars compacta*"

PINK1- PTEN-induced kinase 1

LRRK2 - Leucine-rich repeat kinase 2

ATP13A2 - ATPase type 13A2

E3 - Ubiquitin ligase

ADo - Autosomal dominant

AR - Autosomal recessive

NAC - Non-amyloid-β-component

TH - Tyrosine hydroxylase

SNARE - Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

MPTP - (1- methyl-14-phenyl-1, 2, 3, 6-tetrahydropyridine)

DNA - Deoxyribonucleic acid

EGCG - (-)-epigallocatechin-3-gallate

EC - (-)-epicatechin

PVDF - Polyvinylidene Fluoride

BDNF - brain-derived neurotrophic factor

DP – Doença de Parkinson

IPG - Immobilized pH-gradient

Pro-Q DPS - Pro-Q Diamond phosphoprotein stain

SC - Synthetic complete

MBA - Membrane blocking agent

TBS -Tris-buffered saline

PGK - Phosphoglycerate Kinase

HRP - Horseradish peroxidase

NL - Non linear

SD - Standard deviation

GAL - Galactose

GFP - Green fluorescent protein

RS - Resuspension Buffer

MW - Molecular Weight

SIRT-1- Sirtuin 1

ATG - Autophagy-related protein

mTOR - Mammalian target of rapamycin

Goals

Neurodegenerative diseases are multifactorial and currently there is no effective therapy against the progressive neuronal death characteristic of these pathologies. It is therefore very important to find new therapeutic strategies that can prevent and / or delay the neurodegeneration process. Plant polyphenols appear in response to this demand, having been reported to have substantial neuroprotective activity.

S. cerevisiae is one of the most versatile biological systems used as a model for the study of neurodegenerative diseases. *C. album* leaf PEFs protective effect was already defined in a yeast PD model.

The main objective of this study is to access the effect of *C. album* leaf PEFs on proteome and phosphoproteome of the yeast model of PD. This analysis will be done using two dimensional gels of proteins and will require optimization of the technique involved. A detailed analyses of the protein profiles obtained using appropriate software for image analysis will allow to find proteins that respond to effect of aSyn expression and to the treatment with *C. album* leafs PEFs. The Identification of those proteins will contribute to the overall understanding of our PD yeast model and neuroprotective effects of *C. album* leaf PEFs.

Polyphenols neuroprotective effect in a Parkinson's disease yeast model: phosphoproteome alterations was a work developed between the Disease and Stress Biology Laboratory (DSB), from Instituto de Tecnologia Química e Biológica (ITQB) – Universidade Nova de Lisboa and Cellular and Molecular Neuroscience Unit, from Instituto de Medicina Molecular, from Faculdade de Medicina – Universidade de Lisboa.

Introduction

- **Neurodegenerative Diseases**

Human neurodegenerative diseases are characterized by the progressive loss of structure and/or function of neurons, leading to their death.

Neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's (AD) diseases account for a significant and increasing proportion of morbidity and mortality in the recent world. The most common risk factor for developing neurodegenerative diseases, is aging. With the rise in human lifespan around the world in the last years, the incidence of neurodegenerative diseases, especially AD or PD, has increased dramatically. United Nations population projections estimate a world population of 400 million people over 80 years by the year 2050. Therefore, it is expected that over the next generation, the percentage of elderly people will double and consequently the proportion of persons suffering from some kind of neurodegenerative diseases.

As the population ages, an improved understanding of these diseases will be vital to developing more effective therapies and combating the staggering personal, social, and economic costs of these diseases[1–3].

- **Parkinson's disease, definition and historical perspective**

PD was first described in 1817 by James Parkinson in his monograph *"An essay on the shaking palsy"*, where he identifies six cases of PD[4–7]. Previously referred to as ''paralysis agitans'', nowadays PD is among the most prevalent neurodegenerative disorders as it affects approximately 6 million individuals worldwide[8,9]. Generally, the onset of PD occurs in patients over the age of 50 years and its incidence slowly progresses with increasing age, affecting about 2% of people over 65 years old[9–11]. Clinical manifestations of PD consists in a series of severe motor defects produced by resting muscle tremor, muscle rigidity, brandykinesia and postural instability[6,8–10]. Brandykinesia refers to slowness of movement, and may significantly impair the quality of life. Brandykinesia is considered to be the main feature and the necessary condition for diagnosis of PD[6–8]. These motor disabilities begin to be felt by patients with about 5-10 years of disease, even when treated with symptomatic medication available[7].

Table 1- Parkinson's disease symptoms (Adapted from reference[6])

Motor Symptoms	Non-Motor Symptoms
• Tremor, Brandykinesia, rigidity, postural instability • Hypomimia, dysarthria, dysphagia, sialorrhoea • Decreased arm swing, shuffling gait, festination difficulty arising from chair, turning in bed • Micrographia, cutting food, feeding, hygiene, slow activities of daily living • Glabellar reflex, blespharospasm, dystonia, strial deformity, scoliosis, camptocormia	• Cognitive impairment, brandyphrenia, tip-of-the-tongue phenomenon • Depression, apathy, anhedonia, fatigue • Sensory symptoms: anosmia, ageusia, paresthesias • Dysautonomia (orthostatic hypotension, constipation, urinary and sexual dysfunction, abnormal sweating, seborrhoea), weight loss • Sleep disorders (behavior disorder, vivid dreams, daytime drowsiness, sleep fragmentation, restless legs syndrome)

All these motor symptoms are thought to arise primarily from the loss of dopaminergic (DA) neurons within the "*substancia nigra pars compacta*" (SNpc), located in mesencephalon[8].

Almost two centuries after the first description of PD, there are only symptomatic treatments available for this pathology, which essentially involves substituting dopamine, or suppressing pathological neuronal oscillations via deep brain stimulation[10,12].

The discovery of levodopa in 1960 revolutionized the treatment of PD. Levodopa has the ability to restore dopaminergic transmission deficiency and provides remarkable symptomatic relief to the vast majority of these patients. This remains the most efficacious agent available for PD treatment. However, we soon learned that after several years of treatment most patients develop involuntary movements termed "dyskinesias," which are difficult to control and significantly impair the quality of life[7,9,13].

As a result, in the recent years efforts have been made to develop new therapeutic approaches to treat PD. Interventions such as stem-cell transplantation and gene therapies have been examined as potential treatments for PD. Although early results indicated that patients may obtain long-term clinical benefit from the intrastriatal transplantation of human embryonic mesencephalic tissue, it later became clear that there are several restrictions to stem cell therapy. These include the limited availability of fetal human mesencephalic tissue and the usually very low percentage of viable transplanted cells[9]. On the other hand, in the case of gene therapy, viral vectors have proven to be an ideal

vehicle to deliver or silence genes of interest in the brain in order to develop genetic strategies. Still none of these approaches could stop the degeneration of dopaminergic neurons[9].

Our capacity to discover new PD treatment is limited for several reasons; one of them relates to the complex and multifactorial etiology of PD, which involves an intricate interplay of a large network of factors: genes, environment and gene-environment interactions, lack of knowledge of the specific molecular events that provoke neurodegeneration and aging[5,7,12].

- **Genetic determinants of Parkinson's disease**

In the last decades the discovery of several genes linked to rare familial forms of PD came to prove the role of genetics in disease development and brought new evidence for understanding the pathological mechanisms of PD. The majority of cases are thought to be idiopathic. Nevertheless, in 5-10% of cases, PD presents as a Mendelian form displaying both recessive and dominant models of inheritance[8,14–16]. Several genetic loci named *PARK* are identified to cause PD (Table 2) These include two autosomal dominant genes, *SNCA* encoding alpha-synuclein (aSyn), and leucine-rich repeat kinase 2 (LRRK2), and four autosomal recessive genes, encoding *Parkin,* DJ-1, PTEN-induced kinase1 (PINK1) and ATPase type 13A2 (ATP13A2)[8,14,15].

SNCA gene point mutations (A53T, A30P, E46K, H50Q and G51D), also assigned as PARK1, as well as duplications and triplications, cause autosomal dominant PD[16–18]. On the other hand *Parkin* function as an E3 ubiquitin ligase by targeting misfolded proteins to the ubiquitin proteasome pathway for degradation. The loss of its E3 ligase activity lead to autosomal recessive early onset PD[15]. PINK1 mutations are the second most-common cause of autosomal recessive PD after *Parkin*. PINK1 is located in the mitochondria and mutations in the kinase domain of the protein lead to a mitochondrial dysfunction and dopaminergic neuronal degeneration, as they result in impaired phosphorylation of its substrates in mitochondria[15,16,19]. Loss of function mutations in the DJ-1 gene are associated with forms of autosomal recessive early-onset Parkinsonism. DJ-1 is thought to protect neurons from oxidative stress by acting as redox-dependent chaperone, preventing misfolding and aggregation of oxidized mitochondrial proteins. Accordingly with this DJ-1 knockout mice presents motor impairments and dopaminergic dysfunction[15,16,20]. Mutations in the LRRK2 gene cause autosomal dominant PD. This

protein contains several domains, including RAS/GTPase, tyrosine kinase, MAPKK and leucine-rich-repeat domain, in which mutations can affect signaling pathways relevant in PD[5]. Finally, mutations in *ATP13A2* cause an atypical form of PD with dementia, named Kufor-Rake syndrome[8].

Locus	Gene	Inheritance	Probable function	Chromosome
PARK1	SNCA (alpha-synuclein)	ADo	Presynaptic protein, Lewy Body	4q21
PARK2	Parkin	AR	E3 ubiquitin ligase	6q25.2-27
PARK6	PINK-1	AR	Mitochondrial Ser-Thr kinase	1p35-36
PARK7	Dj-1	AR	Redox-dependent molecular chaperone	1p36
PARK8	LRRK2	ADo	Leucine-rich repeat containinig kinase	12q12
PARK9	ATP13A2	AR	Neuronal P-type ATPase	1p36

Table 2- Genetic factors with PARK status in PD. ADo, autosomal dominant; AR, autosomal recessive. (Adapted from reference[8,14,15])

- **Hallmarks of Parkinson's disease**

Pathological hallmarks of PD are loss of dopaminergic neurons in "*substancia nigra pars compacta",* and the presence of intraneuronal proteinacious cytoplasmic inclusions named Lewy Bodies (LBs)[5,8,10,11,21–24].

LBs are cellular inclusions that can be visualized by histological analysis and are predominantly constituted by the presynaptic protein aSyn, a small acid protein involved in both sporadic and familial cases of PD[5,7,8,10,12,21–23]. LBs present a spherical structure with more than 15 μm in diameter and have a organized structure containing a dense hyaline core surrounded by a clear halo[7] (Fig.1).

Figure 1: Immunohistochemical labeling of intraneuronal inclusions, termed LBs, in the SNpc dopaminergic neuron. Immunostaining with an antibody against aSyn reveals a LB (black arrow) with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (left image). Conversely, immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the LB (right image). (Adapted from reference[7])

PD is often unremarkable, with mild frontal atrophy in some cases, but there is no significant atrophy of brainstem. However, in the SNpc, it can be found the cell bodies of nigrostriatal neurons and these are projected primarily to the putamen (Fig.2). The loss of these neurons, which normally contain amounts of neuro-melanin, produces the classic neuropathological finding of SNpc depigmentation[7,25]. Sections of the brainstem usually reveal loss of the normally dark black pigment in the SNpc (Fig. 2).This loss of pigmentation correlates with neuronal loss of dopaminergic neurons in the SNpc and noradrenergic neurons in the locus ceruleus[7,25].

Indeed, the spreading of LBs correlates with the progression of the disease. In PD, generally LBs are mainly found at sites of neuronal loss such as the SNpc thus indicating that their presence may be related to nerve cell loss[9,26].

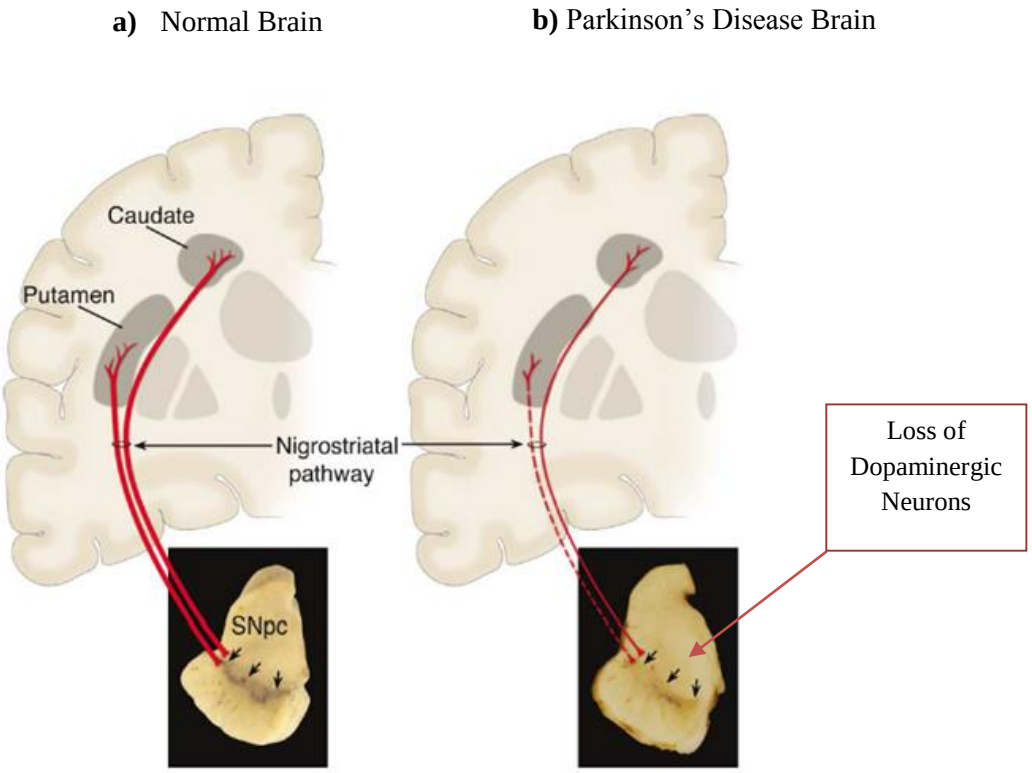


Figure 2: Neuropathology of PD a) Schematic representation of the normal nigrostriatal pathway (in red). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; see black arrows). These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The image demonstrates the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons. **b)** Schematic representation of the diseased nigrostriatal pathway (in red). In PD, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). The image demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; see black arrows) of the SNpc due to the marked loss of dopaminergic neurons. (Adapted from reference[7]).

The aSyn deposition which is the main constituent of the LBs is thought to be central to the pathogenesis of PD. Recent findings have suggested that aSyn deposition at the synapse may be the key issue of the neurodegenerative process. The crucial role of aSyn in PD pathophysiology is further sustained by findings showing that the areas that degenerate in PD (SNpc, striatum and ventral tegmental area) express low levels of aSyn in physiological conditions suggesting that these regions may be more vulnerable to a pathological increase of its levels, which normally occurs during aging[4,9].

- **Alpha-Synuclein: structural and functional properties**

aSyn is an abundant, 140 amino acid long, neuronal cytoplasmic protein. The sequence of aSyn gene (*SNCA*) has a high degree of conservation in numerous organisms, and mutations or multiplications of this gene have been associated with familial forms of PD[4,9].

aSyn is predominantly localized to presynaptic terminals in the central nervous system (CNS), where it is freely associated with synaptic vesicles[5]. It belongs to the synuclein family, which includes also β- and γ-synuclein. These proteins have a common N-terminal sequence containing a different number of repeated regions while they differ in the C-terminal region[4,9].

While all the synucleins are present in human brain, only aSyn is show to be associated with pathological structures in neurodegenerative conditions[27]. Sequence analysis suggests that aSyn consists of three distinct regions: the N-terminal amphipathic region (residues 1-60), the central hydrophobic Non-amyloid-β-component (NAC) region (residues 61-95), and the C-terminal acidic region (residues 96-140)[4,9,22,27].

The N-terminal half of aSyn contains four 11-amino acid imperfect repeats with a highly conserved hexameric motif (KTKEGV), which is involved in the formation of amphipathic α-helices, similar to lipid-biding domain of apolipoprotein-like class A2 and its essential for membrane binding[4,9,27]. Several lines of evidence support the membrane-binding capacity of the N-terminal region. For example, mutations in this region were show to perturb plasma membrane localization of the protein in yeast[27].

This portion of the protein includes the sites of five familial PD mutations: A30P, A53T, E46K and two new mutations discovered H50Q and G51D. Also, N-terminal acetylation is critical for forming α-helical oligomers thus suggesting that this event could have important implications for both the native and pathological structures of aSyn[4,17,18,27] (Fig.3).

The central region comprises the NAC sequence which is highly hydrophobic and aggregation prone. This part of aSyn can undergo a conformational change from random coli to β-sheet structure. Moreover, it is able to form cylindrical β-sheets and amyloid-β-like fibrils and protofibrils. The NAC region is indispensable for aSyn aggregation and toxicity; the deletion of large segments within this motif greatly diminished aSyn oligomerization and fibrillogenesis *in vitro*, and in a cell based-assay. Moreover, mutation of only one single amino acid in this central domain of aSyn can alter the aggregation

properties of the protein, emphasizing the importance of the NAC domain in the aggregation process[4,22,25,27] (Fig.3).

The last segment of aSyn, the C-terminal region (residue 96 to 140) is highly enriched in proline acidic residues and contains three highly conserved tyrosine residues (Y125; Y133; Y136) that are phosphorylation targets. The C-terminal is responsible for the intrinsically disordered nature of aSyn. This domain also plays a regulatory role in the aggregation and fibril formation of the protein, by shielding the hydrophobic NAC domain from the watery solution, in addition, the C-terminal appears to have chaperone activity[4,22,25,27,28].

Deletion or modifications of C-terminal of aSyn, as well as changing the charge or hydrophobicity of the domain, enhances the aggregation rate of aSyn *in vitro* and in cells. For example, phosphorylation of Serine 129 (S129) or nitration of Y125, Y133 and Y136 promote the formation of aSyn filaments or oligomers in the C-terminal half and enhance tendency to aggregate and fibrilize. These results suggest that the C-terminal can play a role of an intramolecular chaperone by preventing aSyn from fibrillization[4,22,25,27,29].

From these characteristics that we see above, it can be inferred that aSyn is a very versatile protein, as it can easily adopt different conformations upon interacting with biological membranes of different compositions, other proteins or protein complexes in physiological conditions[9,22].

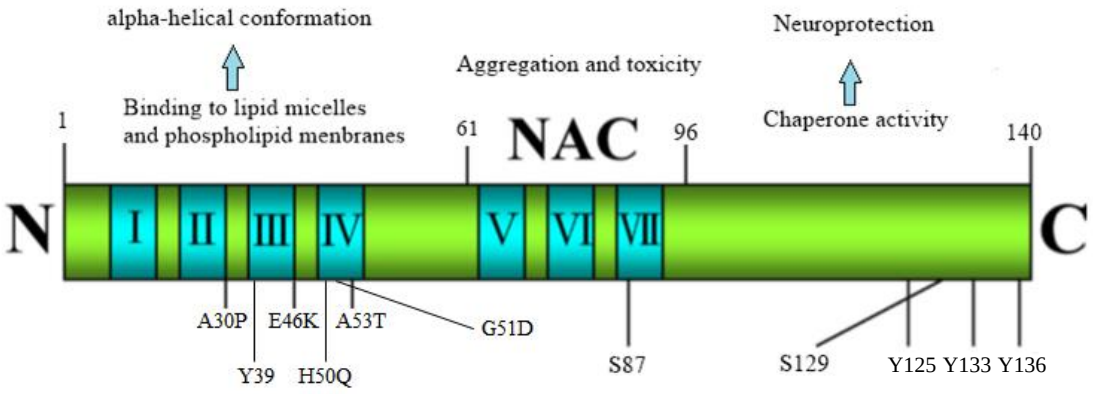


Figure 3: Molecular structure and functional characteristic of aSyn. aSyn is functionally divided into N-terminal (1–60aa), NAC (61–95aa), and C-terminal (96–140aa) domains. The N-terminal domain contains four motifs (blue color) and has five point mutation sites linked to PD (A30P, E46K, H50Q, G51D and A53T) and also a tyrosine residue (Y39) which is a phosphorylation target. The NAC domain, which encompasses the most hydrophobic residues, promotes aggregation, with a phosphorylation site (S87). The C-terminal domain exhibits chaperone activity that tends to decrease protein aggregation, has one phosphorylation site (S129) and three tyrosine residues (Y125, Y133, Y136). (Adapted from[25,27,28])

- **Physiological function of Alpha-Synuclein**

Although aSyn is distributed to almost all subcellular compartments, it is particularly enriched in the presynaptic terminals where it is loosely associated with the distal reserve pool of synaptic vesicles. The high presynaptic concentration of aSyn, and its association with synaptic vesicles, suggest a physiological role of the protein in regulation of synaptic transmission as well as synaptic vesicle recycling[22,25,27,29,30].

Neurotransmitters are stored in vesicles after being synthesized. Those vesicles dock and fuse with the plasma membrane to release neurotransmitters into the synaptic cleft and then restock via recycling from the distal vesicle pool[27,31].

Overexpression, knockdown and knockout of aSyn lead to deficiencies in synaptic transmissions, supporting that it plays an important role in the regulation of neurotransmitter release, synaptic function and plasticity. For example, *SNCA*-knockout mice exhibit an impairment in hippocampal synaptic responses to prolonged trains of high-frequency stimulation that deplete the docked and reserve pool of synaptic vesicles[22]. Moreover depletion and suppression of aSyn induces an impairment of vesicle trafficking between the reserve pool and the ready releasable pool and a deficiency in the neurotransmitter uptake. Experiments with siRNA-mediated knockdown of aSyn resulted in significant decreases in V_{max} for dopamine uptake and the surface density of dopamine transporter in SH-SY5Y cells. On the other hand transgenic mice overexpressing human aSyn display impairment in synaptic vesicle exocytosis and reduction in neurotransmitter release[22,27]. Another study in PC12 cell line show that overexpression of aSyn resulted in an accumulation of vesicles at the synapse[22,27,31], and similar effects have been observed in genetic rodent models of PD[27]. In line with this studies overexpression of aSyn in yeast induces a block of the ER-to-Golgi vesicular traffic leading to defects in endocytosis and exocytosis[4].

In transgenic mice, overexpression of aSyn was found to inhibit tyrosine hydroxylase (TH), the enzyme in charge for dopamine synthesis in dopaminergic neurons and consequently lead to a reduction on dopamine synthesis. In an opposite way down-regulation of aSyn increase TH activity and DA synthesis[27].

The possible role of aSyn in regulating synaptic homeostasis is not exclusively related to its direct interaction with synaptic vesicles, because aSyn is able to interact with proteins that control synaptic vesicle exocytosis, such as phospholipase D2, the family of

RAB small GTPases, the synaptic protein synapsin 1 and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), which are crucial modulators of membrane trafficking, exocytosis and synaptic vesicle release in neuronal cells[4,9].

These data strongly indicate that aSyn displays an important role in the trafficking of synaptic vesicles and in the regulation of vesicle exocytosis and subsequently in neurotransmitter release[4,22]. Thereby, aSyn may contribute to a regulatory effect in the synaptic homeostasis and abnormalities in this regulatory function may influence the neurotransmitter release, leading to alterations on short-term and long-term synaptic plasticity[4,22,27].

- **Alpha-Synuclein Aggregation**

The abnormal deposition of specific proteins in brain tissues is a feature of several age-related neurodegenerative diseases, including PD[7]. Several consequences are hypothesized to be associated with protein misfolding and aggregation: the loss of normal function; gain of toxic function, mechanical disruption of cellular compartments and processes such as synaptic, mitochondrial function and proteasomal activity[12,23,32,33]. The sequence of molecular events that lead to development of neurodegenerative diseases, implicates the accumulation of misfolded protein species, extending from oligomers to higher fibrilar protein aggregates (fig. 4), and once aggregated, these aggregates can grow and enlarge by recruitment of more misfolding proteins. Accumulation of aggregated aSyn affects various functional structures of the nervous system leading to serious cognitive and behavioral alterations[33].

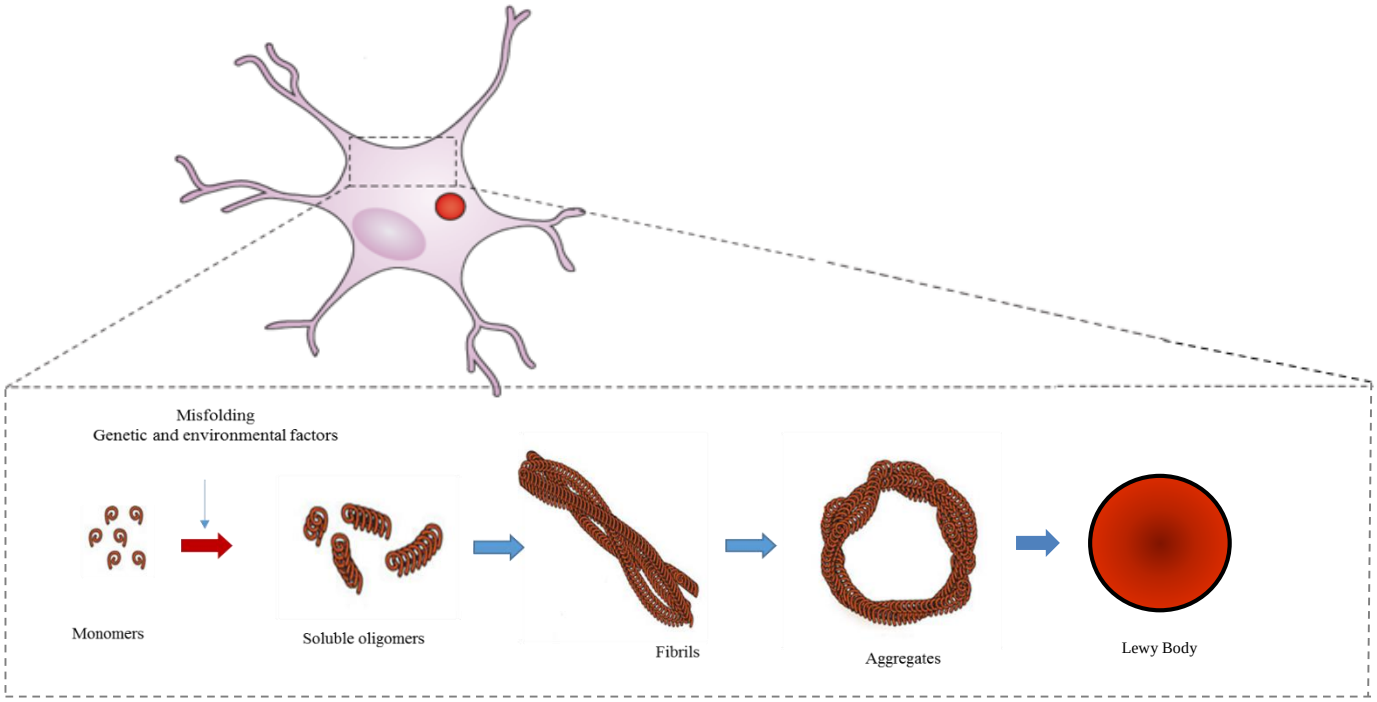


Figure 4: aSyn aggregation. Schematic illustration of the process whereby normal soluble aSyn misfolds is converted into pathological oligomers and higher aggregates that fibrillize and deposit into Lewy bodies in affected neurons in PD brain.

The levels of aSyn in the CNS depend on the balance between the rates of aSyn synthesis, aggregation and clearance. Abnormal protein structure resulting from genetic alterations or overexpression of aSyn could create an imbalance between these mechanisms and will result in abnormal levels of aSyn that might benefit the formation and/or accumulation of oligomeric and fibrillar species, which will ultimately lead to the formation of large aggregates the LBs[8,22,26]. For example, four missense mutations on the gene *SNCA* (A30P, E46K H50Q, and A53T) have been shown to accelerate aSyn aggregation compared to wild-type[17,18,25,29]. These higher rates of aggregation displayed by the mutant forms can be explained by changes in the structure of protein, because these mutations have the ability to disturb the α-helical structure of the protein in the N-terminal region, increasing the possibility of generating pathogenic conformation[25,29]. Many other factors and events have been reported to influence the aggregation of aSyn, such as: i) posttranslational modification of aSyn (phosphorylation; truncation). Phosphorylation of S129 promote the formation filaments and oligomers[27,28]; ii) Incubation of aSyn protein with metals (cooper, iron) and pesticides (paraquat). In yeast, Fe^{3+} ions increase the

number of cells containing aSyn inclusions[34]. Overexpression of aSyn in GT1-7 hypothalamic cells generate aSyn inclusions like structures, and also alterations in mitochondria function, increasing the production of free radicals[29].

Another dysfunction associated with the aggregation of aSyn and its abnormal levels in the brain, is the impairment of the ubiquitin-proteosome system (UPS)[7,8,35]. The UPS is one important pathway for protecting cells against misfolded proteins, clearing misfolded proteins from the cytosol, endoplamatic reticulum and nucleus[36]. Overexpression of wild-type or mutant aSyn in cultures cells and in the brains of transgenic mice is known to impair UPS function. Proteossomal dysfunction and consequent accumulation of misfolded proteins may provoke a vicious cycle, which probably defies the capacity of the proteossomal clearance systems promoting accumulation and development of a self-propagating cycle[7,33,35]. When the misfolded aSyn begin to propagate and reach the acceptor cells, they begin to seeding the aggregation of endogenous protein in a prion–like fashion way[37–39]. This prion-like transmission of aSyn pathology relies on the premise that a sick neuron could release its misfolded aSyn to the extracellular space, or they gain access to the extracellular space when the neuron dies. Once out of the neuron the aSyn could be free to enter an adjacent neuron by endocytosis and act as a template, seeding the aggregation and initiating the formation of a LBs[37]. Studies with neuronal stem cells and embryonic neurons grafted into the brain of aSyn overexpressing transgenic mice can take up host aSyn and develop inclusions, supporting this theory[40]. The accumulation of aggregated aSyn affects various functional structures of the nervous system leading to serious cognitive and behavioral alterations[33].

- **Alpha-Synuclein Phosphorylation**

Posttranslational modifications (PTMs), such as phosphorylation, ubiquitination or sumoylation, which alter the protein size, charge, structure conformation and/or biological function, can also affect protein folding and aggregation, and thereby play a critical role in neurodegenerative disorders[28,41]. Beyond all this PTMs, protein phosphorylation is the most widespread type of modification used in signal transduction affecting every basic cellular process[42].

aSyn protein presents several phosphorylation sites (4 serine, 10 threonine, 4 tyrosine residues) that are highly conserved among numerous species. Nevertheless,

approximately 90 % of aSyn deposited in LBs is phosphorylated at S129. In contrast, only 4 % or less of total aSyn is phosphorylated at this residue in normal brain, suggesting a close relationship between aSyn phosphorylation at S129 and its aggregation[14,41,43–46]. Several studies performed in cell lines associate aSyn phosphorylation with formation of oligomers, cytoplasmic and nuclei aggregates and cytoplasmic inclusions[28], and in transgenic mouse models of PD, aSyn phosphorylation caused accelerated neuronal loss, suggesting a toxic effect of S129 phosphorylation[21]. Even so, some studies in yeast using a S129A mutation that blocks the phosphorylation in S129 residue, resulted in more toxic forms, and increased the number of inclusions and oligomeric species compared with the WT protein[28]. One hypothesis to explain this result lies in the fact that for some proteins phosphorylation acts as a signal for degradation by UPS and/ or autophagy. If this happens with aSyn protein, phosphorylated aSyn might accumulate in LBs due to proteosomal impairment leading to aggregation[28,46].

Multiple kinases have been implicated in aSyn phosphorylation, namely, G-protein coupled receptor kinases (GRK2, GRK3, GRK5 and GRK6), casein kinases 1 and 2 (CK1, CK2) and Polo-like kinase 2 (PLK2) and PLK3[28,44,47]. All these kinases could play an important role in the modulation of aSyn physiology. For example, knockdowns of GKR3, GKR5 and GRK6 significantly decrease levels of aSyn phosphorylated at S129[44]. Overexpression of aSyn increased GRK5 protein expression in SH-SY5Y cells and in brain extracts of transgenic mice expressing human aSyn, additionally GRK5 was found to colocalize with aSyn in LBs of PD patients[28]. Another study showed that the overexpression of PLK2 is protective by mediating selective autophagy clearance of phosphorylated S129 aSyn[48]. This show that these kinases decide the fate of aSyn protein, thus, inhibition or overexpression of relevant kinases could be important in order to generate a potential therapeutic strategy[28,43,48]. Gathering all these data, it is clear that phosphorylation of aSyn is important in the context of its aggregation and toxicity, yet no conclusive agreement of this precise contribution this PTM has toward the disease process. For example, there is still no consensus on whether phosphorylation is neurotoxic or neuroprotective[28].

- **Mitochondrial Dysfunction and Oxidative Stress in PD**

Mitochondria, the power house of living cells and regulators of cell survival and death are especially complex and delicate organelles[19]. The first connection between Parkinson's and mitochondria became apparent in the early 1980s with the discovery that a neurotoxin, MPTP (1- methyl-14-phenyl-1, 2, 3, 6-tetrahydropyridine) causing parkinsonian syndrome inhibits the mitochondrial respiratory complex I (NADH-quinone oxidoreductase)[19,20,40,49]. MPTP is converted to MPP^+ by glial cells in brain and taken up by the dopamine transporter expressed in dopamine neurons. MPP^+ is able to bind and inhibit mitochondrial complex I causing mitochondrial dysfunction and increasing the levels of ubiquitinydated proteins[20,50].

Consistent deficits in the subunits and activity of mitochondrial complex I of the electron transport chain in SNpc of PD patients is a prominent phenomenon. The activity of complex I has been reported to be reduced (in the range of 30%) in the CNS and frontal cortex of PD patients at autopsy. In mitochondrial preparations from PD frontal cortex samples, complex I subunits, derived from mitochondrial genome, were found to be oxidatively damaged[15,40]. Taking in to account this data, aSyn can be found in the mitochondria of the striatum and SNpc of PD patients contributing to impairing the complex I activity[50]. Moreover, in transgenic mouse models of aSyn overexpression, several mitochondrial anomalies were observed. These include oxidation of mitochondria associated proteins, mitochondrial DNA damage, increased oxidative stress, and bioenergetics defects[40]. Oxidative damage to mitochondrial DNA (mtDNA) may compromise encoding of respiratory chain subunits, thus initiating a vicious cycle of oxidative stress and bioenergetics failure[49]. Recently, it has been shown that the N-terminal 32 amino acids of aSyn function as a targeting sequence for import aSyn into mitochondria[20]. This targeting sequence drives aSyn to the inner mitochondrial membrane where it associates with complex I, leading to a decrease in its activity and increasing reactive oxygen species (ROS) production in human dopaminergic neuronal cultures overexpressing wild-type aSyn. In these models overexpression of A53T aSyn enhance these effects (Fig.5)[19,49]. ROS are chemically reactive molecules containing oxygen and are produced in all aerobic cells. Oxidative stress occurs when the generation of ROS overwhelms cells capacity (enzymatic and non-enzymatic antioxidants defenses) to scavenging them[24].

In yeast cells expression of wild-type or A53T mutant aSyn accelerates apoptosis and ROS production. Remarkably these effects did not occur in yeast cells lacking mtDNA. Also, in other model *C. elegans* the overexpression of aSyn reduces the fusion rate of mitochondria[40]. At last, aSyn protofibrils are known to form annular pores in membranes, suggesting that permeabilization of mitochondrial membranes may be involved in aSyn toxicity[20].

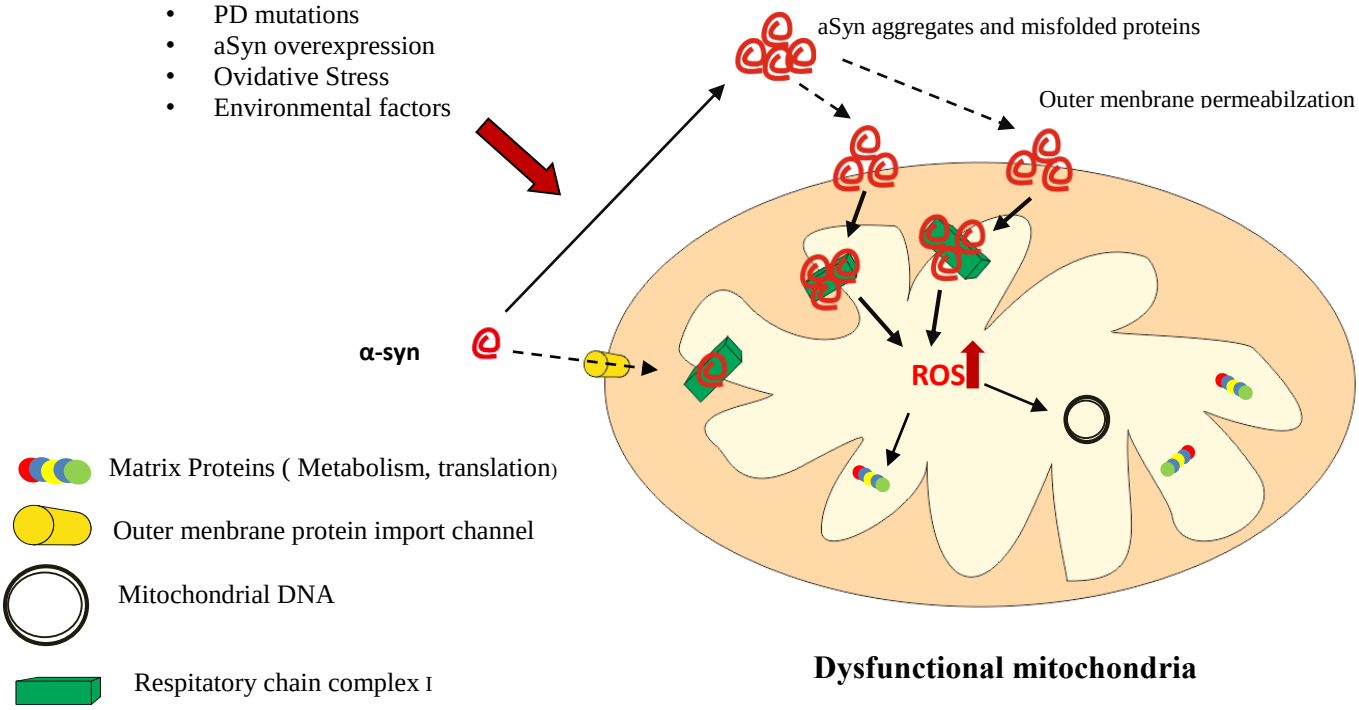


Figure 5: Effect of aSyn in mitochondria. PD mutations; aSyn overexpression; oxidative stress and environmental factors are able to trigger aSyn aggregation and lead to mitochondrial dysfunction. Impairment of mitochondrial complex I activity by aSyn results in an increased production of ROS and promotes aSyn aggregation. Compromising mitochondrial function results in ATP deficits and ultimately will lead to a disruption of cellular homeostasis, abnormal protein aggregation and apoptosis. (adapted from reference[20,49])

As a consequence of impaired electron flux through complex I, mitochondrial ATP production decrease, creating a deficit of energy supply while the generation of ROS is increased[24]. Excessive production of ROS by mitochondria lead to impairment of cellular redox balance pushes the oxidation of biological macromolecules, such as DNA, proteins and lipids leading to failure of biological functions[24,49]. Another study in mice lacking aSyn presented resistance to MPTP, showing that aSyn is an essential mediator of the toxic effects of complex I inhibitors. Remarkably, mitochondrial fragmentation another effect of overexpression of aSyn, can be prevented by PINK1, parkin, or DJ-1 but not by their pathogenic mutants, and the mitochondrial chaperone TRAP1 moderate the aSyn

toxicity in cellular models, supporting the notion that aSyn affects mitochondrial function[40]. Taking in to account all the data above, the association of aSyn with mitochondria complex I raises the possibility that aSyn controls respiratory activity, and may mediate complex I inhibition generating mitochondrial alterations that will lead to an imbalance in cellular oxidative status inducing proteossomal deregulation. This may exacerbate protein aggregation and consequently degenerative events intensifying PD progression[20,50].

- **aSyn the principal player in PD**

At largest level, aSyn and its abnormal accumulation in LBs is a key event in PD[7]. As seen aSyn is an extremely flexible protein that can adopt different conformations and interact with different types of membranes and proteins. aSyn accumulation, pathological modifications, aggregation and transmission can significantly impair synaptic functions causing defects in intracellular traffic and release of dopamine by dopaminergic neurons of the SNpc affecting memory and cognitive function[4,7,51]. Moreover elevated levels of aSyn overwhelm the ability of normal quality-control system to prevent or reverse protein misfolding or eliminate proteins that have assembled into pathological aggregates[5]. Mitochondrial dysfunction and oxidative stress are also characteristics of the abnormal expression and deposition of aSyn, causing deficits in ATP and may also promote misfolded protein conformations. The activation of the programmed cell death machinery are also believed to be factors that start the death of dopaminergic neurons in PD[7,15]. Together PD is an extremely complex neurodegenerative disease associated with different types of cellular dysfunctions.

- **Polyphenols**

Polyphenolic compounds, or polyphenols, are secondary metabolites of plant metabolism and are widely distributed in the plant kingdom. Polyphenols are present in plants, fruits, and vegetables, including berries, olive oil, red wine, and tea[52,53]. They constitute a large group of phytochemicals with more than 8000 identified compounds. Examples of polyphenolic families include, stilbenes, coumarins, phenolic acids and lignans, being, flavonoids are the largest group of polyphenols[53](Fig. 6).

In terms of structure, phenolic compounds are characterized by the presence of at least one hydroxyl functional group (-OH) linked to a benzene ring. (Fig.6) For example, all flavonoids share a basic structure consisting of two or more benzene rings.

The primary functions of these compounds are protection of plants against ROS, produced during photosynthesis[52]. Thus, phenolic compounds like flavonoids have the ability to quenching free radicals and interrupting the propagation of new free radical species[54]. In terms of consumption, the main dietary sources of polyphenols are fruits, vegetables and beverages, and the dietary intake of polyphenols has been estimated at about 1 g/day by Scalbert and Williamson[55,56]. Due to their biological properties including antioxidant, anti-inflammatory, antitumorigenic, antianxiety, anticarcinogenic, antiviral, anti-mutagenic and cardio-protective, polyphenols are currently receiving much attention, emerging as health promoting compounds[55,56]. Several pathways have been reported as being targets of phenolic compounds, thereby demonstrating the broad spectrum of targets and strengthening their usefulness in addressing multifactorial diseases[52,57].

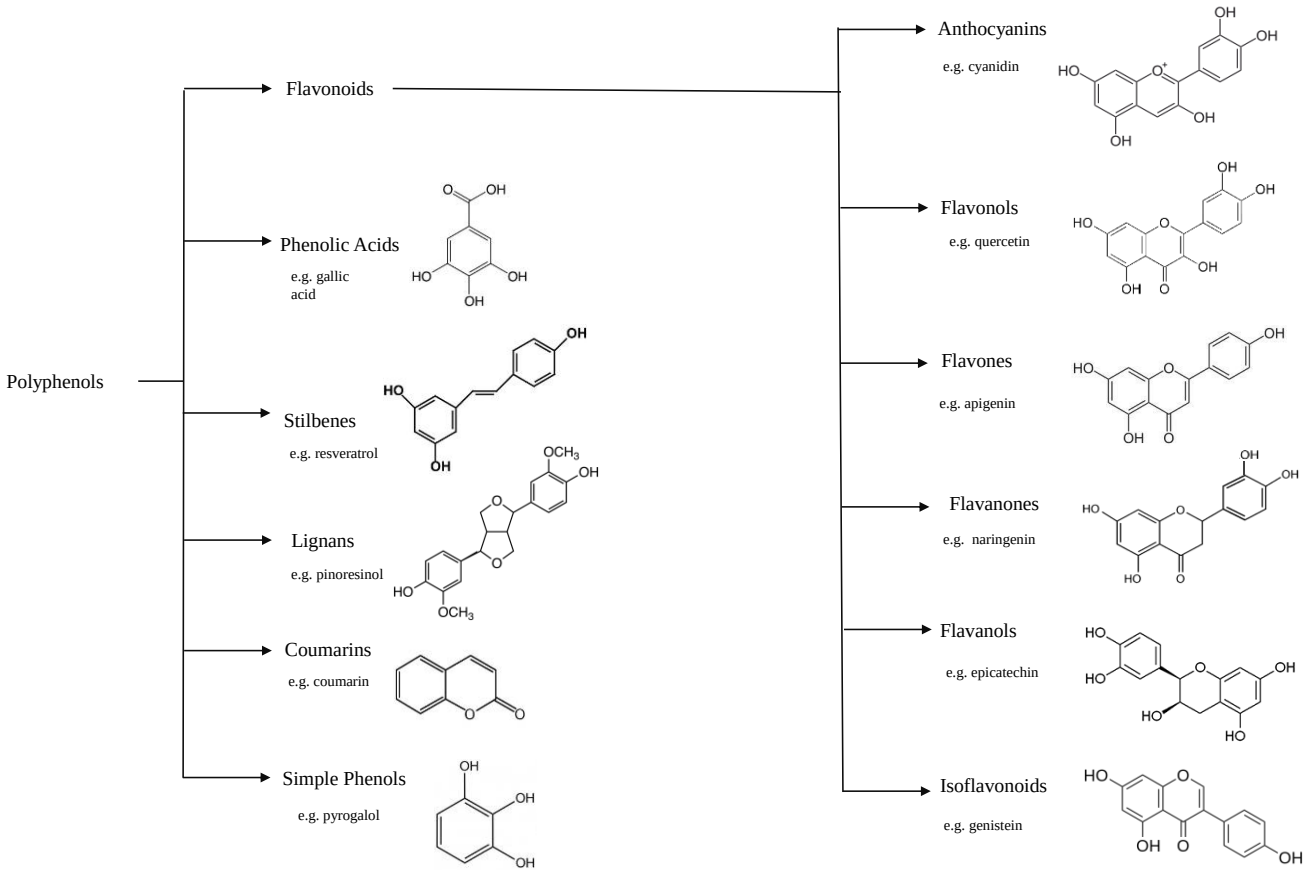


Figure 6: Classification and chemical structures of the main dietary polyphenols classes

- **Polyphenols in Neurodegeneration**

The pathogenesis of neurodegenerative diseases like PD is multifactorial with a complex combination of genetic and non-genetic components. Oxidative damage to neuronal cells, accumulation of iron species in the brain, and reduced or depletion of endogenous antioxidants are examples of pathological aspects of neurodegenerative disorders. Oxidative stress has been related to be an important pathogenic mechanism of neuronal apoptosis in PD[58]. Whereas there is an imbalance in the production of ROS in PD, polyphenols appear as compounds with therapeutic potential, since they are capable scavenge free-radicals in studies *in vitro*[52,59,60]. The neuroprotective effects of many polyphenols and their metabolites rely on their ability to scavenge pathological concentration of ROS and nitrogen species and chelate transition metal ions, breaking the vicious cycle of oxidative stress and tissue damage[61]. For instance, flavonoids, more

precisely catechins and their derivatives, such as (-)-epigallocatechin-3-gallate (EGCG), prevented dopaminergic neurons injury induced by the neurotoxin MPTP in mice. In addition, EGCG protect cells against 6-hydroxydopamine (6-OHDA), a dopaminergic and adrenergic neurotoxin[53]. Moreover, EGCG and quercetin reversed ER-to-Golgi trafficking defects and mitochondrial dysfunction, in yeast, whereas quercetin also showed protective effect in PC12 cells exposed to H_2O_2. Also resveratrol protects neurons against radical overloading and prevents accumulation of ROS[52,62]. Additionally dopaminergic neurons were protected against MPTP when mice received *Ginkgo biloba* two weeks before the neurotoxin been infused[53].

Besides, emerging evidence suggests that the scavenging activity of polyphenols cannot be the only mechanism responsible for their neuroprotective effect, the cellular effects of polyphenols also may be mediated by their interactions with specific proteins central to intracellular signaling pathways, such as MAPK and PI3K/Akt cascades, which regulate pro-survival transcription factor and gene expression[53,57,59]. For instance, *in vitro* studies demonstrate that EGCG could induce MAPKs pathways including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38[53,57]. Activation of ERK is generally pro-survival, while inhibition of c-jun and p38 pathways are also likely to be neuroprotective[57]. It has been indicated that flavonoids are able to block the oxidative stress induced activation of caspase-3 in neurons, via a mechanism involving the suppression of the JNK pathway and downstream partners, supporting the anti-apoptotic action of flavonoids[53,57,63].

There is also data suggesting that fruit and vegetable derived polyphenols are capable of promoting beneficial effects on memory, learning and cognitive performance through their ability to exert effects directly on the brain's architecture for memory[53,57,63]. Fisetin, a flavonoid found in strawberries, has been show to improve long-term potentiation and enhance object recognition in mice by a mechanism dependent on the activation of ERK and the cAMP response element-binding protein (CREB)[57,63]. Equally, the flavonol (-)-epicatechin (EC) induces activation of ERK and CREB in cortical neurons, increasing CREB regulated gene expression[57,59,63]. CREB is a critical transcription factor in the activation of neurotrophins such as brain-derived neurotrophic factor (BDNF), that promote neuronal survival, differentiation, synaptic function and memory[57,63].

The emerging observation is that the intake of polyphenol rich foods, such berries, throughout life holds a potential to limit neurodegeneration or reverse cognitive

performance impairments. Polyphenolic compounds could exert beneficial effects on cells through the modulation of different pathways such signaling cascades and anti-apoptotic processes. However, *in vivo* studies are needed to clarify whether sufficient concentrations of polyphenols, reaches the brain, and alters cell signaling pathways[53,63].

- **The Awesome power of the Yeast tool**

The yeast *Saccharomyces cerevisiae,* also known as baker's or budding yeast, is the most extensively studied eukaryotic organism and has been used as a biotechnological tool for many centuries. Yeast cells are widely used as model organism because basic cellular mechanisms, such as cell division, DNA replication, metabolism, protein folding, membrane trafficking and protein quality control mechanisms are well conserved between yeast and higher eukaryotes, including mammals[10,64]. Yeast cells exhibit several characteristics that make it an incredibly useful model for genetic and biochemical approaches. These includes simple manipulation and screen for induced phenotypes, rapid growth on defined media with a doubling time of approximately 90 min. Survives indefinitely in frozen glycerol stocks, inexpensive when compared with other eukaryotes, easy replica plating, and the most important, highly versatile DNA transformation and the ability to integrate genes by homologous recombination[10,64,65]. As result of the advantages described above, *S. cerevisiae* was the first eukaryote organism to be fully sequenced in 1996 and of the 6000 genes predicted to be encoded by its 12000 kb genome, approximately 80% are functionally characterized[65]. Altogether, these qualities make the yeast an attractive model to modeling and study human brain disorders, for example[10,66]. Two different strategies can be implemented when modeling a human disease in yeast. If the gene implicated in the disease has a yeast homolog, it is possible to study its function directly. If on the other hand, the gene underlying the disease is absent in yeast, it can still be modeled via the heterologous expression of the human gene in yeast cells[10]. As it happens with PD and other neurodegenerative diseases for example[30].

Despite all the potential of yeast, biological pathways and mechanisms present in higher eukaryotes are absent in yeast and, on the other hand, some pathways, such cell wall biosynthesis, have no counterpart in mammals. Therefore, all findings in yeast related to human diseases must ultimately be validated in more physiologically relevant systems prior to their trial in humans[64,65].

- **Parkinson's disease Yeast Model**

Regardless of its limitations as a unicellular eukaryote, yeast expressing aSyn can faithfully reproduce key features of PD pathology. Since the initial description of the first yeast model of PD by Outeiro & Lindquist in 2003[30], other groups have taken advantages of the potential that the yeast model offers to study PD related features. One example, are the yeast strains carrying the galactose-inducible aSyn constructs, where aSyn is expressed under the control of a galactose-inducible promoter. (Fig. 7)

Several characteristics of PD can be reproduced in yeast based on the heterologous expression of human wild-type aSyn and clinical mutants. Accordingly studies using this models linked the formation of aSyn aggregates directly to toxicity. In addition, the expression of aSyn wild-type and the mutant A53T inhibits growth and promote cell death in a concentration-dependent manner[10]. These observation are reviewed in other models such as rat primary mesencephalic cultures and *Drosophila*. Furthermore the impairment of vesicular trafficking, endocytosis and vacuolar degradation are also effects of aSyn on yeast[10,67]. The ER-to-Golgi transport and secretory pathway are blocked and impairment in UPS system is also reported models expressing aSyn. Treatment with ferrous ions triggered an increase in oxidative stress associated with enhanced inclusion formation and toxicity of aSyn. Yeast was also used for drug screens in order to identify several therapeutic compounds that rescue aSyn toxicity[10,34].Namely, the flavonoids, quercetin and (-)-EGCG, were found to counteract aSyn toxicity, reducing damage to mitochondria[10,34,52,58,63]. Collectively, these data reveal a phenotype based yeast model that recapitulates pathological properties of human aSyn[34]. In conclusion, it is clear that yeast represents a valuable cellular tool decipher the devastating pathological role of aSyn in PD[10,65,67].

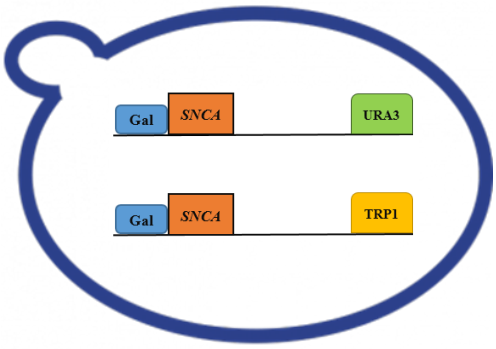


Figure 7: Example of Yeast Construction to study PD related features. Yeast model carrying two integrative plasmids, containing two copies of *SNCA* gene (aSyn) with a galactose inducible promoter and auxotrophic markers *URA3* and *TRP1* respectively, which by homologous recombination are integrated into the host genome.

- **Proteomics approaches**

Proteomics seeks to study all proteins expressed in any given organism, whether by abundance, activity, structure, posttranslational state or other modification, protein interaction with each other in complexes and signaling pathways. Several technologies can be used in the proteomic research. For example with the introduction of two-dimensional gel electrophoresis (2-DGE) in the 70s made it possible to obtain a picture of the cellular protein content, the proteome[68,69]. The proteome is defined as the set of proteins expressed from the genome of an organism at a given time under defined conditions. Generally, the proteome is modified according to the specific biological and pathophysiological state of the organism[70].

Proteome analysis are carried out by combining techniques of protein separation and identification. Among the proteomic tools, can be highlight the electrophoretic techniques, and in terms of identification, stands out mass spectrometry (MS) as technique of choice. 2-DGE is a powerful separation technique which allows simultaneous resolution of hundreds or even thousands of proteins.

This technique separates proteins by their isoelectric point (p*I*), that corresponds to the specific pH which the net charge of the proteins is zero, through an immobilized pH-gradient (IPG) gel matrix in the first dimension, and then by molecular weight (mW), the second dimension[70–73]. Visualization of proteins in gels is accomplished with staining techniques. Colloidal Coomassie blue and zinc are examples of stains for the visible

range, in terms of fluorescence staining SPYRO ruby is an option. Each technique presents specific aspects, sensitivity, and reproducibility. Protein stains must be compatible with MS[72,73]. Gel images can be examined visually, but objective spot detection, quantification and comparison of a large number of protein spots require a computer support, through the use of specific image analysis software that allows a precise examination of the proteome[72]. Image-analysis software also guides the excision of proteins from gels for further analysis and characterization.

There has been rapid growth over the last decade in the field of proteomic, strongly motivated by improvements in MS and informatics platforms. Without doubt, proteomics has already provided significant insights into biological function of proteins and this will carry on as the technology continues to improve, the ultimate goal, is to obtain full coverage of all proteins in proteome-wide studies, allowing a complete separation and characterization of all protein species present in a given organism[69,71,72].

- **Phosphoproteomics**

Protein phosphorylation is the most widespread and diverse of the PTMs found in nature, playing a crucial role in the regulation of many biological functions in prokaryotic and eukaryotic cells[74–76]. Phosphorylation and dephosphorylation on serine, theorine, and tyrosine residues are the key regulators of cellular signaling pathways, which are involved in most of cellular events and biological process, like protein synthesis, gene expression, cell survival and apoptosis[74,76]. Phosphorylation affects protein structure by changing the charge and hydrophobicity, thus, conformational changes can be induced, affecting the properties of the protein, leading to activation, deactivation, or change in protein interaction partners[75,76]. It has been reported that up to 50% of all proteins may be phosphorylated during lifetime, and over 100 000 potential sites of phosphorylation exist in the human proteome[75].

Thereby, analysis of phosphoproteomes and its alterations is increasing and gaining more attention among researchers.[74] In order to respond to this increased interest in the study of phosphorylation, phosphoproteomics, appears as an emerging field. With the aim of characterizing phosphoproteins and phosphorylation sites, mapping phosphorylation networks and understand protein phosphorylation events controlling various cellular processes[74]. One of advances in this area is the development of a unique

fluorescence stain, Pro-Q Diamond phosphoprotein stain (Pro-Q DPS)[77,78]. Specific for phosphoproteins, Pro-Q DPS made possible the selective detection and quantification of the phosphoproteome, following separation by 2-DGE[71,77]. Pro-Q DPS binds directly and specifically to the phosphate moiety of phosphoproteins independently of which amino acid residue is phosphorylated and is compatible with other stains, such as glycoprotein and total protein stains and MS[71,75–77]. For example, a combination of Pro-Q DPS and SPYRO ruby or CCB for staining all proteins in the same 2-DGE can be apply. This approach allows the quantitative detection of different classes of proteins in 2-DE gels[71]. Since the deregulation in phosphorylation is a characteristic of some neurodegenerative diseases, such as PD and AD, thus phosphoproteomics might be the technique of choice to unravel the protein's phosphorylation and interaction in networks both in normal and diseased state[74,75].

Materials and Methods

- **Plant Material**

Leaves from *C. album* (*Corema album* (L.) D. Don) were selected for this study. Leaves, collected by random sampling in Comporta (southern region of Portugal), were frozen in liquid nitrogen and stored at -80 ºC. Before utilization the samples were ground, freeze-dried and subsequently stored at -20 ºC.

- **Extract Preparation**

Secondary metabolites were extracted from *C. album* leaves using clean solvents, as previously described by Tavares and co-workers[79]. Briefly, to 1 g of fresh weight plant material, 6 ml of hydroethanolic solvent 50 % (v/v) (ethanol/water) were added. The mixture was shaken for 30 min at room temperature in the dark and then centrifuged at 12400 *g* for 10 min at room temperature. The supernatant was first filtered through a paper filter and then trough a 0.2 μm cellulose acetate membrane filter. The resulting extracts were freeze-dried and stored at -20 ºC.

- **Extract Fractionation**

The hydroethanolic extracts were fractioned by solid phase extraction (SPE) using Giga tubes 2g/12mL, C18-E units (Phenomenex®), according to Tavares and co-workers[79]. This procedure allows the removal of interfering molecules like organic acids, sugars and to concentrate into a polyphenol enriched fraction (PEF) Briefly, the column was first equilibrated with acetic acid (Panreac; Spain) in water 0.5 % (v/v) (CH_3COOH/H_2O) and the dried extracts were ressuspended in the same equilibration solution, before being applied to the columns. The unbound material, which contained interfering molecules, was washed out using two volumes of the same solution. Finally, the bound fraction, that is enriched in polyphenols, was eluted with a solution of acetic acid in acetonitrile (Riedel-de Haën®; USA) 0.5 % (v/v) (CH_3COOH/CH_3CN). Fractions were concentrated in a Spped-Vac (Labconco, USA) and stored at -80 ºC.

- **Measurement of Total Phenol Content**

Determination of total phenolic compounds was performed by the Folin-Ciocalteau method, adapted to a 96 microplate reader, as described by Tavares and co-workers[79]. Briefly, to each well of a microplate, 235 µL water, 5 µL sample, 15 µL Folin-Ciocalteau reagent (Fluka®; USA) and 45 µL saturated Na_2CO_3 (Riedel-deHaën®; USA) were added. The microplate was incubated for 30 min at 40 ºC and the absorbance at 765 nm was measured. Gallic acid was used as the standard and the results were expressed in mg of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} dw) of plant material. All measurements were performed with three replicates.

- **Yeast strains**

In this study *Saccharomyces cerevisiae* was used as a model organism. The selected strains were aSyn-1, aSyn-2 (*MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1 GAL1pr-syn WT::TRP1; GAL1pr-syn WT::URA3*) and Control strain (*MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1 TRP1 URA3*) encoding two copies of aSyn the hallmark protein of Parkinson disease, under the control of a galactose inducible promoter and the respective empty vectors, respectively. These strains were isogenic to W303 (*MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1).*

- **Media and growth conditions**

The strains were grown and maintained on YPD (Yeast extract, Peptone, Dextrose) solid medium [1 % (w/v) yeast extract (HiMedia; India), 2 % (w/v) glucose (Sigma Aldrich; USA), 2 % (w/v) peptone (HiMedia) and 2 % (w/v) agar (HiMedia)] at 4 ºC, after growth at 30 ºC for 48 hours. For long term storage, YPD cultures containing 50 % (v/v) glycerol were frozen at -80 ºC.

For all experiments, an overnight pre-inoculum was prepared in synthetic complete (SC) liquid media [CSM (complete supplement mixture) 0.79 $g.L^{-1}$ (QBiogene), 0.67 % (w/v) YNB (yeast nitrogen base) (Difco; USA) and 1 % (w/v) raffinose (Sigma Aldrich, USA)]. Subsequently, yeast cultures were diluted in the same medium, in order to synchronize the cultures and grown overnight at the same conditions until a final optical density at 600 nm ($OD_{600\ nm}$) of 0.7-0.8 ± 0.1. Cells were diluted to a final $OD_{600\ nm}$ of 0.2

in SC liquid medium supplemented with 2 % (w/v) galactose (Sigma Aldrich) supplemented or not with *C. album* leaf PEF and grown under agitation during 6 hours at 30 º C. After this period yeast cell cultures were ready for the subsequent analysis. Cell density of cultures was measured using the plate spectrophotometer Power Wave XS (Biotek®).

- **Growth Curves**

aSyn cytotoxicity as well PEF toxicity and cytoprotection was evaluated by means of growth curves. After inoculum preparation and 6 hours of induction with 2 % (w/v) galactose or 2 % (w/v) glucose (Sigma Aldrich, USA), cells were diluted in the same media to a final $OD_{600\ nm}$ of 0.03 in a 96 well plate supplemented or not with *C. album* leaf PEF to a final volume of 100 µL. For PEF toxicity and protection evaluation, were used several concentrations of PEF (0, 15, 30, 62.5, 125, 500 µg GAE. mL^{-1}).

The cultures were incubated at 30 ºC for 24 hours, with constant shaking and yeast growth was kinetically monitored hourly by $OD_{600\ nm}$ readings. All experiments were performed with three biological replicates.

- **Phenotypic Growth Assays**

In a 96-well plate, cells were diluted to an $OD_{600\ nm}$ of 0.2 in SC liquid medium containing 2 % (w/v) galactose or 2 % (w/v) glucose and supplemented with *C. album* leaf PEF (0, 15, 30, 62.5, 125, 500 µg GAE.mL^{-1}). The microplate was incubated during 6 hours at 30 ºC, with constant shaking. Subsequently, 30 µL of yeast cells were withdrawn to a new 96-well plate and the $OD_{600\ nm}$ was adjusted to 0.1 with PBS (phosphate buffer saline) pH 7.4. Finally, a four-fold serial dilutions were made in PBS pH 7.4 and 5 µL was spotted onto galactose and glucose supplemented solid medium, plates were incubated for 48 hours at 30 ºC. Experiments were perform with at least three biological replicates. Images were acquired using Quantity-one® software from Chemidoc and obtained in the end of 48 hours of growth.

- **Protein extraction**

Cells were diluted to a final $OD_{600\ nm}$ of 0.2 in SC liquid medium with galactose 2 % (w/v) supplemented or not with 30 μg GAE.mL $^{-1}$ of *C. album* leaf PEF and grown as described in section 2.6. Cells were harvested by centrifugation (2500 *g* for 5 min at 4 ºC), washed with PBS and cell pellets were immediately stored at -80 ºC. Protein extraction was performed according to Santos *et al*[66]. Briefly, cell pellets were thawed on ice and ressuspended in lysis buffer [Tris-HCl 25 mM, pH 7.4 (Carl Roth®; Germany) supplemented with 1 % (v/v) cocktail inhibitor proteases III (Merk Milipore; USA)]. Cell lysis was carried out with six consecutive steps of vortexing and ice cooling with glass beads (425–600μm) (Sigma Aldrich, USA). The mixture was centrifuged at 15,300 *g* at 4 ºC for 5 min, the supernatant was collected and the pellet was ressuspended in lysis buffer. The mixture were centrifuged in the same condition, the supernatant collected was mixed with former. The mixture was clarified by centrifugation at 15,300 *g* at 4 ºC for 10 min.

- **Protein Quantification**

Protein quantification was performed using a modified Lowry's method as reported by Bensadoun and Weinstein[80]. The original method has been modified so that proteins can be assayed in the presence of interfering chemicals such as Tris or ammonium sulphate. The absorbance of the samples was measured at 750 nm[80,81].

- **SDS-PAGE**

Ten μg of protein were subjected to SDS-PAGE in 10 % (w/v) acrylamide gels with 1 mm thickness at 200 V during 1 hour in electrophoresis buffer [25 mM Tris base (Carl Roth®), 192 mM glycine (Carl Roth®) and 0.1 % SDS (Merk Milipore)]. The Protein Marker VI (AppliChem®; Germany) was run along with the samples.

- **Western Blot**

The electrotransfer of polypeptides was done according to Swerdlow *et al.*[82]. After SDS-PAGE, gels were incubated in electrotransfer buffer [25 mM Tris base, 19 mM glycine, 0.1 % (w/v) SDS and 20 % (v/v) methanol pH 8.3] during 15 min. PVDF membranes (Amersham Biosciences, Sweden) were incubated in methanol followed by incubation in electrotransfer buffer. The gel and the membrane were assembled in transfer cell (Trans-Blot electrophoretic transfer cell, BioRad, Amadora, Portugal) containing transfer buffer. Transfer was carried out at 70 V for 1.5 hours at 4 ºC.

- **Immunodetection**

After protein transfer, membranes were dried at room temperature and blocked with TBS-Tween-MBA [5 % (w/v) membrane blocking agent (MBA; GE Healthcare®), prepared in Tris-Hcl 1.5M pH 7.8, and Tween 20® (TBS-Tween)] for 30 min with agitation at room temperature. The membranes were first incubated with the primary antibody anti-aSyn (C-20)-R (1:500) (Santa Cruz Biotechnology, USA) in TBS-Tween-MBA overnight at 4 ºC. The membranes were then washed three times for 5 min in TBS-Tween followed by incubation with secondary antibody Goat anti-Rabbit (1:300) (Milipore) in TBS-Tween-MBA for 2 hours at room temperature. For loading control, the membranes were incubated with primary antibody PGK (1:3000) (Life technologies) in TBS-Tween-MBA for 2 hours and after three washes of 5 min in TBS-Tween the membranes were incubated with secondary antibody goat anti- Mouse HRP conjugated (1:5000) (Pierce) in TBS-Tween-MBA for 2 hours at room temperature. All incubation were performed with constant orbital agitation. Antibody detection was performed with chemiluminescent substrate (FemtoMax Super Sensitive Chemiluminescent HRP Substrate, Rockland Inc., Gilbertsville, USA). Images were acquired using the Molecular Imager ChemiDoc XRS (Quantity One© software v. 4.6.6; BioRad, Amadora, Portugal).

- **Sample preparation for Two-Dimensional Electrophoresis**

Before 2D SDS-PAGE analyses cell extracts were subjected to a cleaning process using the 2D Clean-Up kit® (GE Healthcare®; Sweden) according to the manufactures instructions. This kit is designed to clean samples from impurities such as nucleic acids, lipids and salts that would otherwise produce poor 2-D results due high levels of interfering substances or low concentration of proteins. After the cleaning process, protein pellets were resuspended in ressuspension buffer [7 M Urea (Carl Roth®), 2 M Thiourea (Sigma Aldrich), 4 % (w/v) CHAPS (Merk Milipore), 2 % (v/v) IPG Buffer (1:2, 3-10 NL:4-7L) (GE Healthcare®), 1 mg. mL^{-1} DNase (Merk Milipore), 60 mM DTT (Carl Roth®), 1 % (v/v) cocktail inhibitor proteases III] and stored at -20 ºC until further use.

- **Two-Dimensional Gel Electrophoresis**

The isoelectric focusing electrophoresis (IEF) was performed according to Santos *et al.*[66] using the IPGphor™ system (GE Healthcare®) and IPG strips with non-linear gradient gel of pH 3-10, 13 cm (IPG strips Immobiline DryStrips, GE Healthcare®). Briefly, the samples containing 75 μg of protein were centrifuged at 13,400 *g* at room temperature during 5 min and the supernatant was applied to the Strip Holder. The IEF was carried out at the following conditions: 30V for 12 hours, followed by 250V for 1 hour, 500 V for 1.5 hours, 1000V for 1.5 hours, 2500 V for 1.5 hours, 8000V for 1 hour gradient and 8000V for 4 hours at 20 ºC. At the end of the IEF the strips were stored at -20 ºC until second dimension separation was performed[66].

Prior to SDS-PAGE, the IPG Strips were equilibrated in two sequential incubations of 15 min in buffer solution [50 mM Tris-HCl (pH 8.8), 6 M urea, 2 % (w/v) SDS, and 30 % (v/v) glycerol (Sigma Aldrich)]. DTT at a concentration of 0.06 mM was added to first the equilibration solution and 0.135 mM iodoacetamide (Sigma Aldrich) to the second one. The equilibrated strips where subjected to SDS-PAGE in 12.5 % (v/v) acrylamide gels with 1 mm thick. Protein standards (PeppermintStick™ Phosphoprotein molecular weight; Molecular Probes®, USA) were run along with sample at 15 mA per gel for 15 min and then 30 mA per gel for 4 hours.

- **Pro-Q® Diamond**

For phosphoprotein detection, after electrophoresis, protein polypeptides/spots were stained with Pro-Q® Diamond phosphoprotein gel stain (Molecular Probes®; USA) according to Santos *et al*[66]. Briefly, the gels were placed in a fixing solution [50 % (v/v) methanol (Carlo Erba Reagents) and 10 % (v/v) acetic acid] overnight with orbital agitation. After the fixation step, the gels were washed three times with ultra-pure water for 15 min and were incubated with Pro-Q® Diamond solution for 2 hours in the dark with orbital agitation. After this period, the gels were incubated for 30 min in a solution containing 20 % (v/v) acetonitrile and 50 mM sodium acetate pH 4 (Riedel-de Haën®). This step was repeated twice and were followed by three washes for 5 min in ultra-pure water, the gels were immediately scanned in a laser imager with 532-nm excitation and 580 bandpass emission filter FLA-5100 Fuji Photo Film Co, Ltd.

- **Commassie Brilliant Blue G**

For total protein detection Commassie Brilliant Blue G (Amersham Biosciences, Sweden) was used. Staining was done according to Neuroff *et al.*[83]. Briefly, the gels were incubated first in a solution containing 34% (v/v) methanol, 17% (w/v) ammonium sulphate (VWR) and 2% (v/v) of phosphoric acid (Carl Roth®) during 1 hour. After this period, a solution containing 1.1% (w/v) Commassie Brilliant Blue G and 34% (v/v) methanol was added and gels stayed in this solution for 48 hours with orbital agitation. Before image acquisition the gels were washed three times for 15 minutes. Gel images were acquired by the Image Scanner (Amersham Biosciences, Sweden).

- **2D Gel Analysis**

For spot detection, measurement and matching, gel images were analyzed in Progenesis SameSpots software (Nolinear Dynamics, Newcastle, United Kingdom). The first step in the analysis was image control. Images went through a process of image quality assessment for accurate image analysis. The next step was to select a suitable reference image to align all the images and then the areas of the gel to be excluded from

the analysis were defined. Once made the detection of spots they were edited, validated and reviewed. One of the crucial steps in this analysis is spot volume normalization: the intensity of each spot in gel is expressed as a proportion of the total protein intensity detected in the entire gel. Individual spots with irregularities (i.e. Ink spikes) were rejected.

Results and discussion

- **Establishment of the Yeast model of PD**
  - **Growth curves**

Saccahoromyces cerevisiae strains that were selected for this study are isogenic *to W303 (MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1).* The aSyn-1 and aSyn-2, that were tested are both with the same genotype *(MAT alpha can1-100 his3-11 15 leu2-3 112 ade2-1 GAL1pr-syn WT::TRP1 GAL1pr-syn WT::URA3),* encoding two copies of *SNCA* gene (aSyn) under the control of a galactose inducible promoter and finally, the control strain (*can1-100 his3-11 15 leu2-3 112 pRS304::TRP1 pRS306::URA3 ade2-1).* These strains were used to evaluate the effect of aSyn expression in cell phenotype.

The most common method to study cell growth is through batch culture, in liquid medium, allowing the monitorization of yeast growth and the detection of phenotypic changes or growth defects based on the genetic mutation presents in strains. In a classic cell growth analysis in liquid medium, the number of yeast cells in a growing population is monitored by the optical density at 600 nm ($OD_{600\,nm}$) and pertinent growth parameters, such as *Lag* phase time, doubling time (DT) and final biomass are extrapolated from the growth curve. *Lag* phase time is determined based on the linear regression of log growth phase equation obtained after logarithmic transformation of $OD_{600\ nm}$ values, and corresponds to the time required by cells to respond to environment changes. The DT is also obtained based on the logarithmic transformation of $OD_{600\,nm}$ values. DT can be used to characterize the kinetic growth of a population and is an indicator of the microorganism response to a specific condition. Growth rate varies typically with the strain and is deeply influenced by the growth medium, being obviously higher under an optimal growth environment. The rate of growth together with stationary-phase OD increments reflects quantitatively the minor changes in growth phenotype and is show by cell density or final biomass in the end of growth[10,84,85].

In order to determine growth curves and parameters of yeast expressing or not aSyn, as a first approach in this optimization, yeast cells of each strain were grown in SC medium with glucose (aSyn "off") or galactose (aSyn "on") as described in sections (Media and growth conditions; Growth curves) in material and methods. The *SNCA* gene is under the control of a galactose promoter, which is regulated by the carbon source present in the growth media. Thus glucose represses expression of aSyn, and galactose

allows the expression of aSyn protein. The growth was monitored hourly during 24 h (Fig. 8a, b).

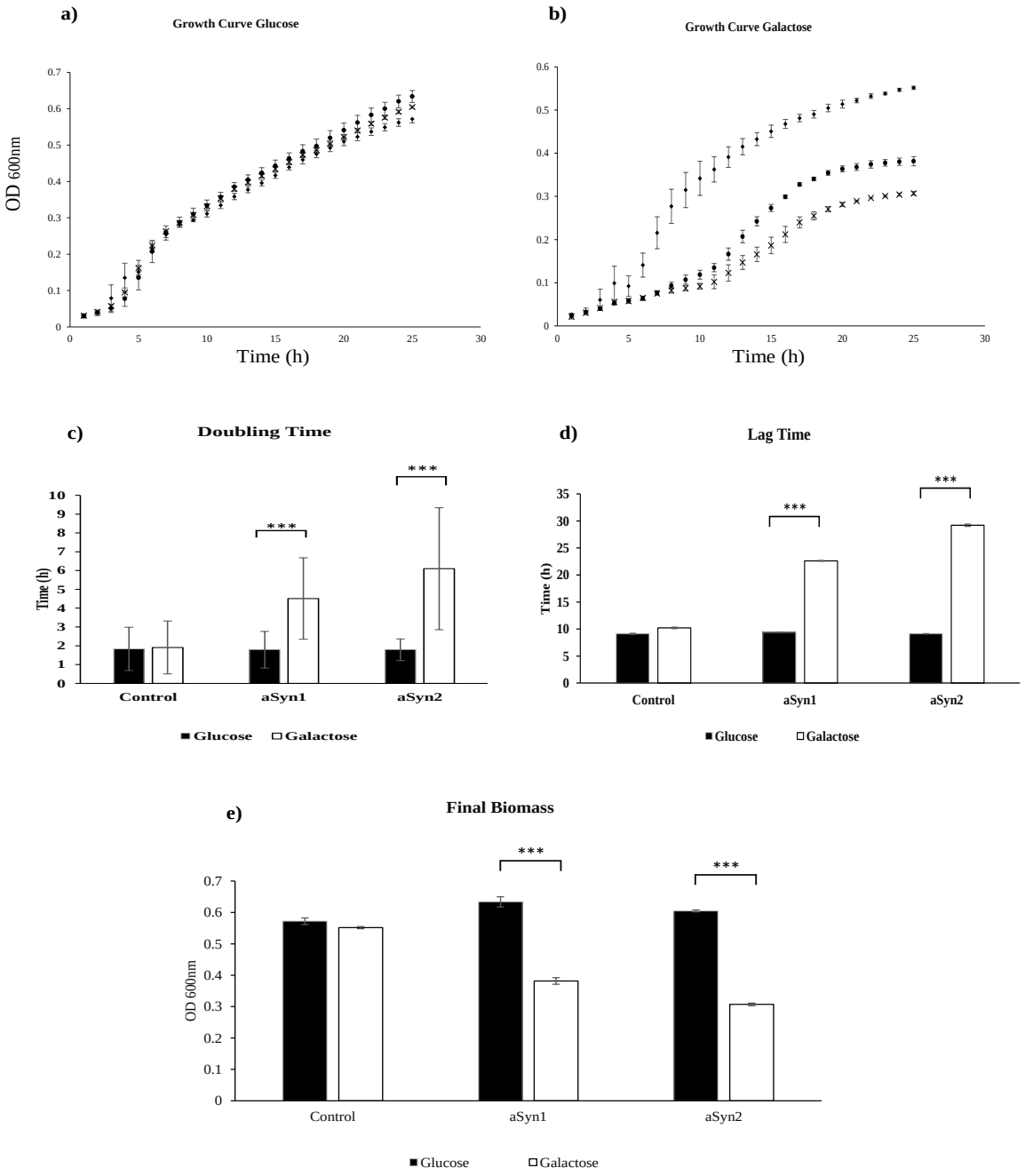


Figure 8: Growth Curves and growth parameters of *S. cerevisiae* strains expressing or not aSyn. *S. cerevisiae* strains Control (♦); aSyn-1 (●); aSyn-2 (×) were grown in SC liquid medium with **a)** glucose or **b)** galactose for 24h at 30ºC. **a)** and **b)** Grown was kinetically monitored hourly by OD measurements at 600 nm and growth curve represented. **c)**; **d)**; **e)** Growth parameters were determined based on the growth curve, **c)** doubling time, representing the time necessary to cells to duplicate, determined by logarithmic transformation of raw OD 600nm from growth curves. **d)** *Lag* time, representing the adaptation time, determined by logarithmic transformation of raw OD 600nm from growth curves. **e)** Final Biomass (OD 600nm), representing the final OD of each culture, is calculated based on the measurements of OD at 24 hours of growth. Results represent the mean ± SD of three independent biologic replicates. *** indicate statistically significance between Control, aSyn-1 and aSyn-2, for a $p<0.001$ respectively.

The results of growth curves (Fig 8a, b) show that the three strains, control; aSyn-1; aSyn-2, grew equally in medium with glucose as carbon source as expected (Fig 8a). On the other hand, yeast strains aSyn-1 and aSyn-2 show a decrease in growth compared to control strain, when grown in medium containing galactose as carbons source (Fig. 8b). This is due to the fact that aSyn expression is toxic to cells. The aSyn protein is the main component of Lewys bodies, the pathological hallmark of PD. This is the first effect of the expression of aSyn protein, leading to a reduction in growth rate, compared with the control strain (Fig. 8b).

Nevertheless, aSyn-2 displays a lower growth rate when compared to aSyn-1(Fig. 8b). It was noted that aSyn-1 and aSyn-2 display a much higher doubling time when compared with empty strain in medium with galactose (Fig. 8c). In contrast, in medium with glucose, where there is no expression of aSyn protein, the DT of all the strains are quite similar (Fig. 8c). Consistent with these results, the *lag* time of aSyn-1 and -2 are greatly higher compared with the empty in galactose. As expected in glucose the lag times are identical (Fig. 8d).

Finally, in terms of final biomass, it is possible to observe that control cells exhibit similar growth on both glucose and galactose medium. The same is not true for aSyn-1 and -2, since we found that this two strains have a lower final biomass in SC medium with galactose, compared with growth on glucose (Fig. 8e). Looking at the data of the growth curves and growth parameters, it was determined that aSyn-2 reveals a DT and a lag time higher compared to aSyn-1 (Fig. 8c, d).

Globally, the results observed reflect the toxicity of aSyn for yeast cells and are in agreement with what was previously reported (Outeiro and Lindquist, 2003). Moreover, the results show that aSyn-2 strain is more sensitive to the toxic effects of aSyn expression than aSyn-1 strain. Thus, this strain was selected for the further studies. Nevertheless, these results indicate a toxic effect associated with the expression of aSyn protein, which is reflected in the final biomass, lag time and DT of cells.

Also, the optimal galactose concentration to induce aSyn expression in our yeast cells were accessed by immunoblotting, as shown in annex 1.

- **Effects of polyphenols on yeast growth**

Phytochemicals are known for their antioxidant activities and, among these, polyphenols are recognised for their neuroprotective effects. These phytochemicals occur naturally in plants and current evidence strongly supports a contribution of polyphenols to the prevention of several diseases like cardiovascular diseases, cancer, and osteoporosis.

In this work it was analysed the effect exerted by polyphenol enriched fraction (PEF) from leaves of *Corema album* in yeast cells expressing aSyn protein.

Corema album (L.) D. Don, belong to *Empetraceae* family, also known as Portuguese crowberry (Fig. 9), is a dioecious shrub endemic from Iberian Peninsula and Azores Islands that grows in sandy dunes and coastal cliffs of the Atlantic coast. It produces edible berries, which look like little pearls. This fruit is not currently commercially explored and very few studies were performed, still this plant can be a potential source of phytochemicals. Leaves were used in this study, based on previously data obtained by DSB lab (unpublished data), indicating that the leaves reduced the percentage of yeast cells displaying aSyn inclusions compared with other matrixes, such as fruit.

Figure 9: *Corema album* (Portuguese Crowberry; Camarinha), fruits and leaves

After collected the plants, the next objective was to obtain the polyphenol enriched fractions from the leaves, in which the total content of these metabolites is concentrated. Solid phase extraction with C18-E columns was used for this purpose (section Extract Preparation in material and methods) allowing also the removal of organic acids, sugars and minerals that otherwise could interfere with the following analysis. After this concentration step, the total phenolic content of several fractions was estimated by Folin-Ciocalteau method (section Measurement of Total Phenol content in material and methods). Therefore, control and aSyn-2 yeast cells were incubated with several concentrations of *C.album* leaf PEF (0, 15, 30, 62.5, 125, 500 μg GAE.mL $^{-1}$), and the

growth was kinetically monitorized for 24 hours (section Growth curves in material and methods).

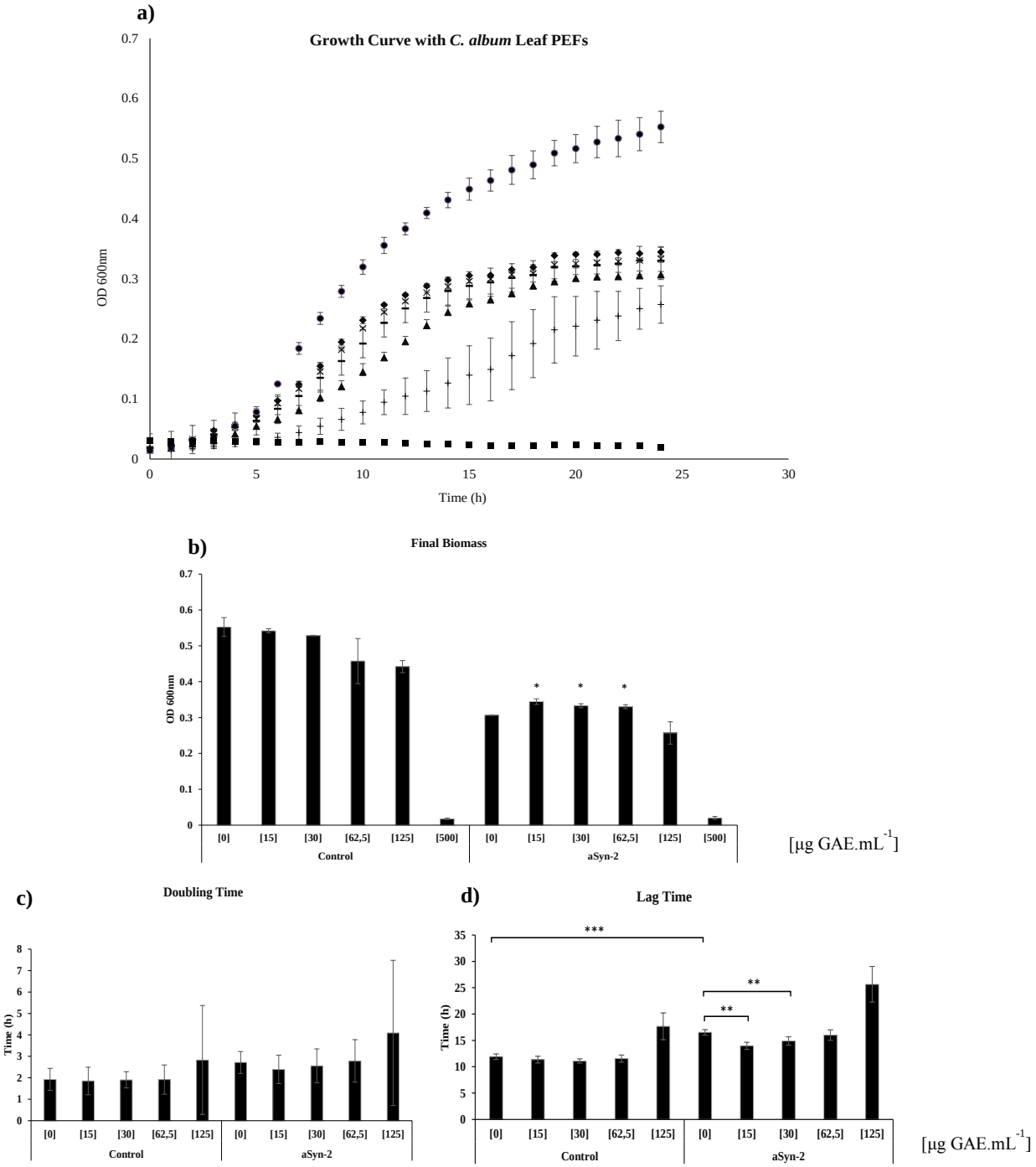


Figure 10: Growth Curves and growth parameters of *S. cerevisiae* strains (Control and aSyn-2) with *C. album* leaf PEFs. *S. cerevisiae* strains incubated with different concentrations of *C. album* leaf PEFs (µg GAE.mL^{-1}) Control [0] (●); aSyn-2 [0] (▲); aSyn-2 [15] (♦); aSyn-2 [30] (×); aSyn-2 [62.5] (-); aSyn-2 [125] (+); aSyn-2 [500] (■); Yeast cells were grown in SC liquid medium with galactose for 24h at 30ºC. **a)** Grown was kinetically monitored hourly by OD measurements at 600 nm and growth curve represented. **b)**; **c)**; **d)** Growth parameters were determined based on the growth curve. **b)** Final Biomass (OD 600nm), **c)** Doubling time, **d)** *Lag* time. Results represent the mean ± SD of three independent biologic replicates. *, ** and *** indicate statistically significance between Control and aSyn-2, for a $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

Considering the data from the growth curve and final biomass, 125 and 500 μg GAE.mL $^{-1}$ are the most harmful to yeast, affecting the growth of the both strains (Fig. 10a, b).

Lag time and DT are also affected for 125 and 500 μg GAE.mL $^{-1}$of *C. album* leaf PEF (Fig. 10c, d), since 500 μg GAE.mL $^{-1}$ is very toxic, it was not possible to calculate the value of *lag* and DT for this concentration (Fig. 10b).

Interestingly, some concentrations of PEF increase the final biomass, of aSyn-2 cells comparatively with cells that were not incubated with PEFs, namely, 15, 30, 62.5 μg GAE.mL $^{-1}$ (Fig. 10b). At the same time, it was observed that *lag* time is reduced by 15 and 30 μg GAE.mL $^{-1}$ (Fig. 10d). In contrast, the DT of cells incubate in presence of PEFs did not change significantly relative to untreated cells, (Fig. 10c). Furthermore, considering the results of the *lag* time and DT was determined that expression of aSyn seem to affect more the replication mechanisms of yeast cells, than the time for adaptation to the new conditions.

To summarize, with this methodology it was possible to determine some concentrations of *C.album* leaf PEF that reduce the toxicity of aSyn and improve cell viability. Thus, our results show *C. album* leaf polyphenols as potential neuroprotective agents, with beneficial effects against aSyn toxicity. Emphasizing, the neuroprotective potential of *C. album* leaf PEFs, previous studies of DSB lab (unpublished data) with a different yeast model (p426 GAL-aSyn WT::GFP) reveal a protective effect against aSyn toxicity. Again *C. album* leaf polyphenols enhance the viability of yeast cells expressing WT aSyn. These results are very interesting, because in both models with different features *C. album* leaf PEFs are able to counteract the aSyn induced toxicity.

- **Phenotypic Growth assays with polyphenols**

Phenotypes are used to determine the sensitivity of cells to a given condition. Plating or spotting assays on solid media can be used to track the growth of cells treated with toxic agents for example, or to screen non-toxic concentrations of chemicals that affect the viability. Spot assays involve the visual inspection of colony size, number and the thickness of cells, which allows a visual evaluation of colonies growth under a given condition and showing just the cells that are still capable of replication. Consequently, spot assays are used as methodology to access cellular viability[84,85].

In order to evaluate how aSyn expression affected the normal growth of the strains and to gain further insight into the protective effect and toxicity of *C.album* leaf PEF, growth phenotypic assays, on solid medium, were performed. Control and aSyn-2 strains were incubated with several concentrations of *C.album* leaf PEF (0, 15, 30, 62.5, 125, 500 μg GAE.mL $^{-1}$). Strains were grown as described in the section (Media and growth conditions) in material and methods, and subsequently grown for 6 hours in SC medium with galactose or raffinose supplemented with *C.album* leaf PEF. At the end of this incubation cells were spotted on galactose and glucose solid medium, and incubated for 48 hours at 30ºC (section Phenotypic Growth Assays in material and methods) (Fig. 11a).

The results demonstrate that some concentrations of *C. album* leaf PEF are able to counteract aSyn toxicity, in two different approaches. (Fig. 11b, c). First, in aSyn-2 cells grown in SC medium with raffinose, where there is no expression of aSyn, incubated with various concentrations of PEF and then placed on solid media containing galactose, allowing the expression of aSyn protein. We detect a protective effect of several *C. album* leaf PEF (Fig. 11b), namely 15; 30; 62.5 μg GAE.mL $^{-1}$. In contrast cells that were not treated with PEFs presented a reduced grown (Fig. 11b), emphasizing the toxic effect of aSyn, leading to inhibition of yeast growth in aSyn-2 strain. In medium with glucose both control and aSyn-2 strain show similar growth rates (Fig. 11b). These results indicate that preconditioning with PEFs can increase the viability of yeasts expressing aSyn. Similar assays (Fig. 11c) were performed, where cells were incubated with PEFs for 6 hours in SC medium with galactose. Therefore, expression of aSyn was induced in the presence of PEFs. Still, 15, 30, 62.5 μg GAE.mL $^{-1}$ of *C. album* leaf PEFs were able to rescue cells from aSyn toxicity. Nevertheless, 30 μg GAE.mL $^{-1}$of *C. album* leaf PEF showed the best protection, enhancing the growth of aSyn-2 yeasts (Fig. 11b, c). Control and aSyn-2 yeast cells that are spotted in glucose solid medium display a normal phenotype, as expected (Fig. 11c). PEFs were toxic at concentrations of 125 and 500 μg GAE.mL $^{-1}$ (Fig. 11b, c), like it was already detected in the growth curves.

In conclusion, it was observe a protective effect of *C. album* leaf PEF against aSyn toxicity and induced death. Some concentration of PEF reveal improvements in growth of yeast cells expressing aSyn protein. Consistent with this result, yeast cells expressing aSyn who did not receive treatment with polyphenols failed to grow or form colonies.

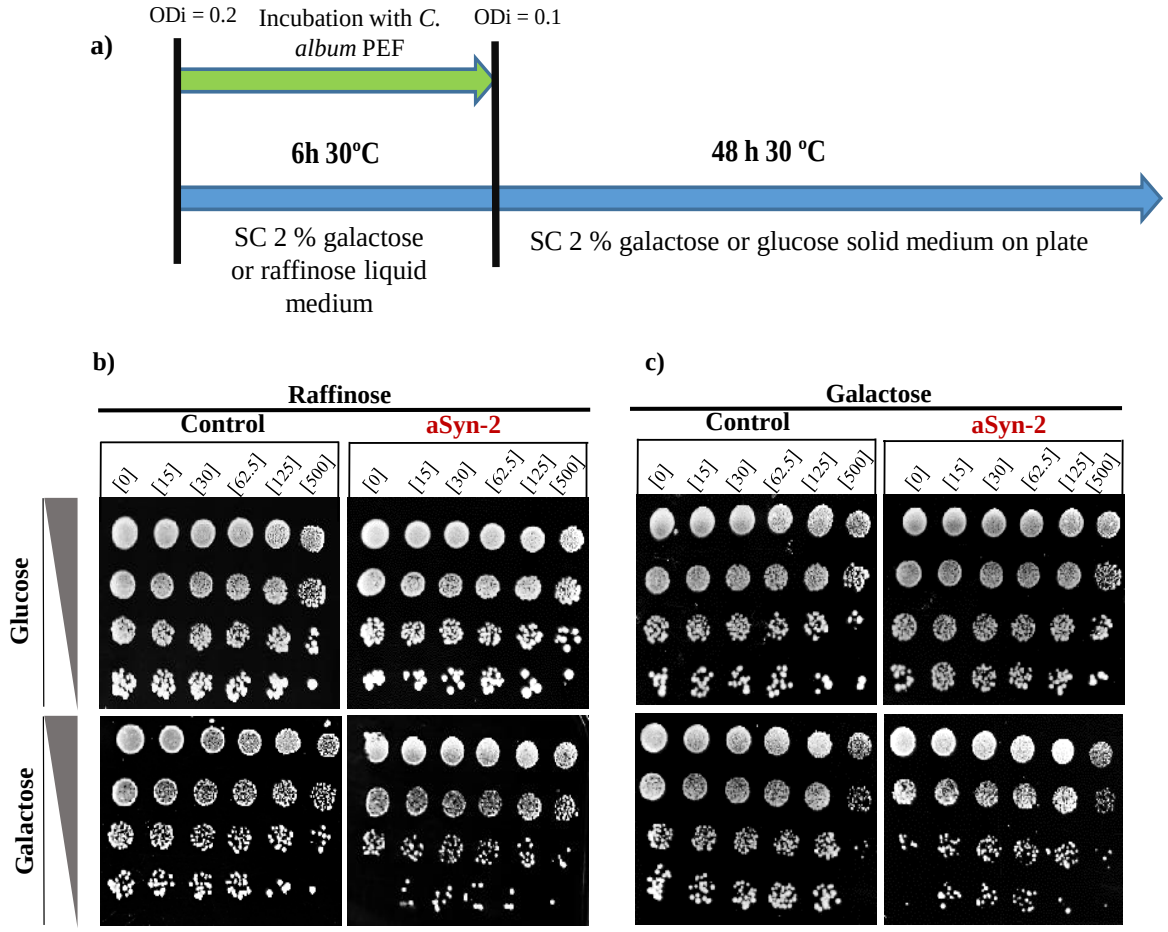


Figure 11: Phenotypic growth assay of control and aSyn-2 strains grown in the presence of polyphenol fractions. a) Schematic representation of cells growth for 6 hours at 30 ºC, with constant shaking in liquid medium containing galactose or raffinose, with several concentrations of PEFs. Cells were serial diluted, subsequently 5 µL of each dilution was spotted in solid medium with glucose or galactose and incubated for 48 hours at 30 ºC. **b)** Spot assays for control and aSyn-2 strains that grew 6 hours in SC liquid medium containing **raffinose**, supplemented with several concentrations of *C. album* leaf PEFs. Spotted in solid medium with galactose or glucose and incubated for 48 hours at 30 ºC. **c)** Spot assays for control and aSyn-2 strains that grew 6 hours in SC liquid medium containing **galactose**, supplemented with several concentrations of *C. album* leaf PEFs. Spotted in solid medium with galactose or glucose and incubated for 48 hours at 30 ºC. Image acquisition was made with Chemidoc XRS and Quantity-one software and the most representative of biological replicates is show.

Overall, these results suggest that *C. album* leaves PEF is an efficient cytoprotective agent in the yeast model of PD. This PEF counteracted the cytotoxicity induced by aSyn expression. Also in a different yeast model, from DSB lab, expressing WT aSyn, *C. album* leaf PEFs improve the growth phenotype of WT aSyn comparing to yeast cells not treated with PEFs (unpublished data). Additionally, based on the results from the growth curves and the spot assays, 30 µg GAE.mL $^{-1}$ of *C. album* leaf PEF as defined as the suitable concentration for further proteomics approaches.

- **Proteomic Approach**
  - **2DE protocol optimization**

Before proceeding with proteomic analyses an optimization step was required. The aim of these optimization procedures was to preserve as much quality and quantity of protein samples during the extraction process, as well as to remove interfering in samples. During the protein clarification and extraction process, proteins can easily become unfolded, denatured, or damaged. Thus, an appropriate buffer solution need to be added to a protein mixture through the extraction process, in order protect the integrity of the proteins while separating them from other cell components. Therefore one of the improvements that were evaluated consisted in the use of various extraction buffers, in particular, Tris-HCL 25 mM, pH 7.4 and ressuspension buffer (RS)[7 M Urea, 2 M Thiourea, 4 % (w/v) CHAPS, 2 % (v/v) IPG buffer, 1 mg. mL^{-1} DNase, 60 mM DTT, 1 % (v/v) cocktail inhibitor proteases III]. Beyond this optimization, another variant was studied, more precisely the use of cleaning protein kit, the 2D Clean-Up kit. Protein samples frequently contain interfering agents, like ionic detergents, metal ions, salts, lipids, charged polysaccharides, peptides, enzyme substrates, and other agents with charge. These agents often lead to poor performance on 2 DE gels, resulting in smeared or low resolution gels. Thus, it can be important the use of such kits to remove interfering agents and concentrate our samples. For these optimization processes it was used a different yeast strain, the wild type *S. cerevisiae* BY4741. The protein extraction was performed as described in the section (Protein extraction) material and methods, using different buffers with and within cleaning step. At the end of the extraction it were obtained four samples, (Tris; Tris Clean; RS; RS Clean), two corresponding to each of the sample buffers (Tris and RS), one to be quantified, and the other was exposed to a cleaning process with 2D clean-up kit (section Sample preparation for Two-dimensional Electrophoresis in Material and methods).

Then, 75 ug of protein of each sample were subjected to a two dimensional gel electrophoresis (2-DGE) (section Two-Dimensional Gel Electrophoresis). For protein detection, the gels were stained in Commassie Brilliant Blue G for 48 hours with orbital agitation (section Commassie Brilliant Blue G in Material and methods).

The first information found from these assays is related to the amount of protein obtained at the end of extraction. Extraction performed with Tris-HCl yielded about 2 $\mu g/\mu L^{-1}$ protein. Still, the combination of Tris-HCl extraction followed by a cleaning step with 2D clean-up kit, leads to an increase of the concentration of protein (4 $\mu g/\mu L^{-1}$).

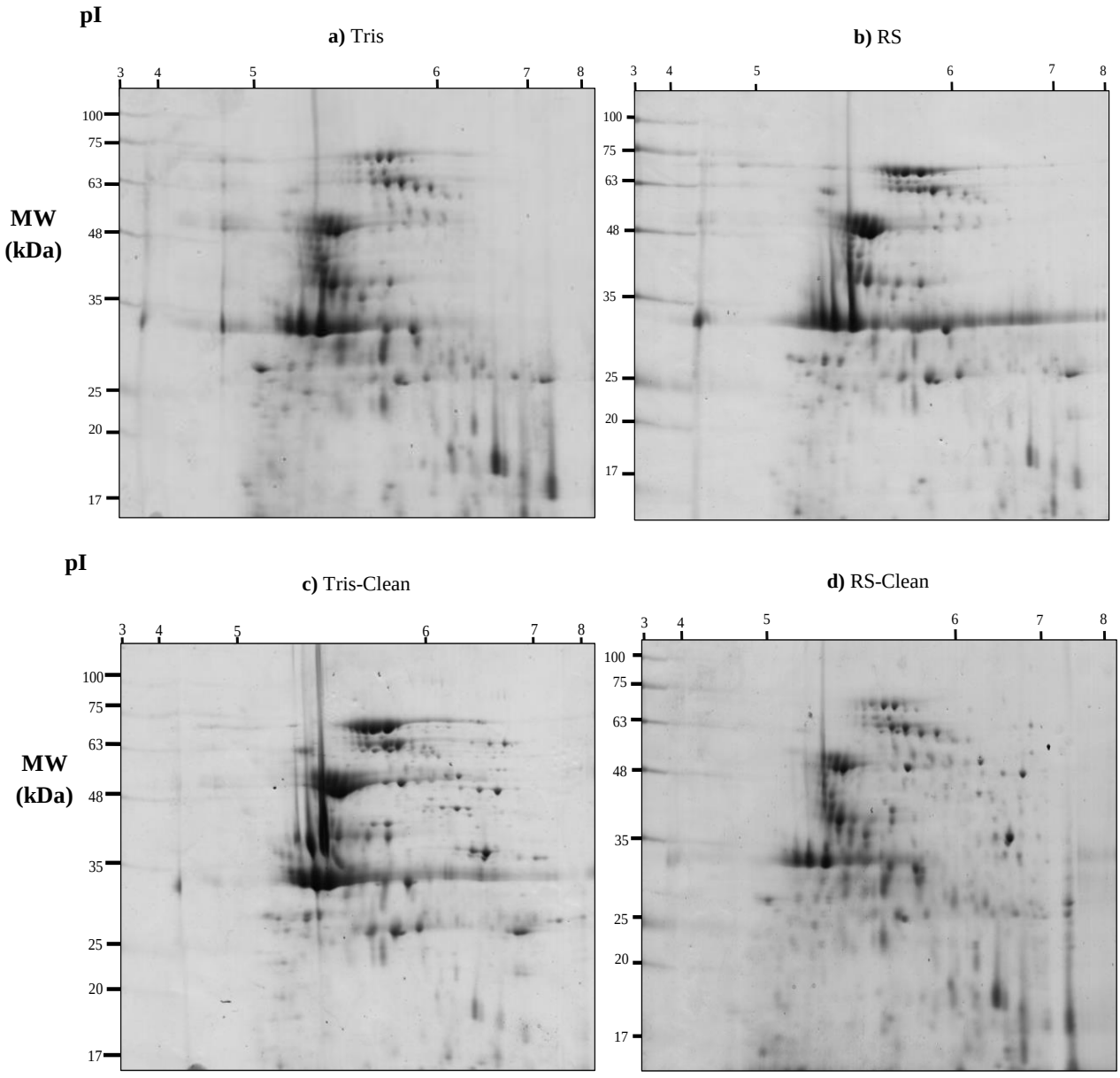


Figure 12: Representative 2-DGE gels of BY4741 strain cell proteome (75 µg). The gels were CBB stained. **a)** Extraction performed with Tris-HCl buffer. **b)** Extraction was done with RS buffer. **c)** The extraction was performed with Tris-HCl buffer, and the protein sample was submitted to cleaning step using 2D Clean-Up kit. **d)** Extraction was performed with RS buffer, with subsequently cleaning step using 2D Clean-Up kit. IEF was performed with 3-10 NL IPG strips.

In extraction procedure with RS buffer, the amount of protein obtained was approximately 4 $\mu g/\mu L^{-1}$ with the cleaning step this value remained unchanged. Thus, with both buffers, Tris-HCl or RS, it was obtained similar amounts of protein. However, the cleaning step appears to be important in the extraction with Tris-HCl, once the

cleaning step increased the final protein concentration. Contrary to extraction with RS, where cleaning the sample did not increase the amount of protein.

In addition, through these optimization steps it was possible to observe that the quality of our gels varies with the type of buffer is used, and if the sample underwent cleaning process or not (Fig. 12). For instance a clear difference between the gels from protein extracts that were cleaned (Fig. 12c and 12d), compared to the gels with the same sample without cleaning step was observed (Fig. 12a and 12b). In Tris Clean gel (Fig.12c) it is possible to observe a greater amount of spots, these are better separated and with a much higher resolution compared with Tris condition (Fig. 12a) Similarly, the same happened in RS gels (Fig.12b, 12d), where it can be observed that cleaning also allowed to obtain a greater number of spots mainly in the alkaline part of the gel with a better separation (Fig. 12d). The overall quality of protein separation and spot resolution using 2D Clean-Up Kit has been shown to be superior to the samples that did not undergo the cleaning process (Fig. 12a and 12b). Preparation of protein samples with the kit reduces horizontal streaking, improves spot resolution, and increases the number of spots detected. However, between the two procedures (Fig. 12c, 12d) it is possible to conclude that protein gels performed with samples extracted with Tris-HCl and clarified with 2D Clean-Up Kit present a higher spot resolution and definition when compared to the RS Clean. Although both samples have been cleaned, the quality of the gels is not similar. This difference could be related to the composition of buffers.

The RS buffer is more efficient probably due to its chaotropic agents, such as urea and thiourea allowing a high degree of solubilization of many cellular components, but it may be able to extract some components which are of no interest, thus increasing the burden of interfering present in the sample, hampering the efficiency of the 2D clean-up kit. Taking this into account, the extraction with Tris-HCl buffer combined with the use of 2D clean-up kit seems to provide gels with more quality and accurate results. Thus this procedure has been implemented in all proteomic approaches in this work.

- **Proteome Analysis**
  - **Protein pattern of Control and aSyn-2 yeast strains with *C. album* leaf PEFs**

Since aSyn is the principal component of LBs, and considering the protective effect of *C. album* leaves PEF against aSyn toxicity, in this work it was investigated the cellular responses to expression of aSyn protein and the mechanisms underlying the

beneficial effects of *C. album* leaves PEF. To address these questions, we used a quantitative proteomics approach based on 2-DGE to screen differentially expressed proteins. In order to quantitatively investigate proteome changes of yeast cells in response to aSyn expression and the effect of the incubation with *C. album* leaves PEF, it was analysed and compared the proteomes of control and aSyn-2 strains. Both strains were grown as mentioned in section (Media and growth conditions) material and methods, supplemented with 30 µg of *C. album* leaf PEF, the ideal concentration based on the results of the growth assays. After protein extraction and cleaning process (section Sample preparation for two-dimensional electrophoresis in material and methods) four different protein samples were collected: control; aSyn-2; that were grown without PEFs and control+PEF; aSyn-2+PEF that grown in the presence of PEFs. Therefore, 75 µg of each protein sample were submitted to 2-DGE (section Two-Dimensional Gel electrophoresis in material and methods). The resulting 2-D gels were stained for total protein detection (section Commassie Brilliant Blue G) and compared to identify differentially expressed proteins. The results are shown in figure 13.

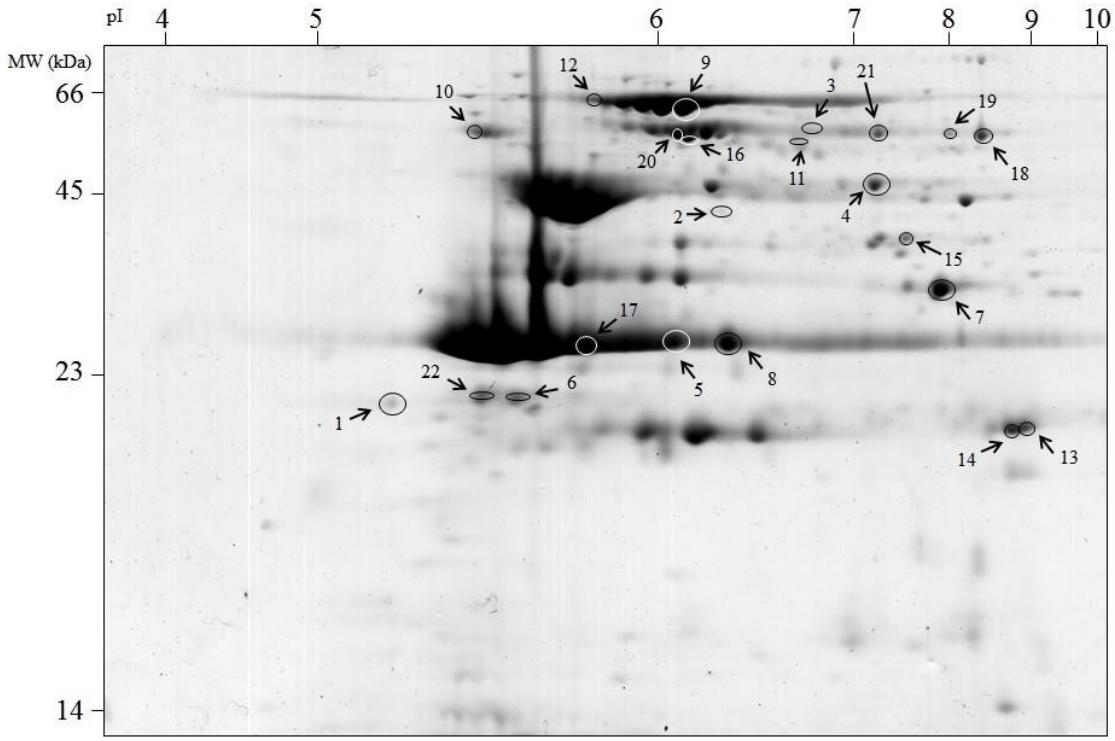
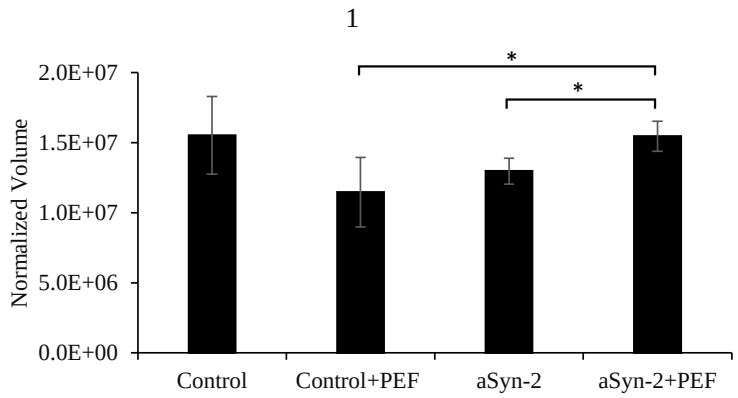
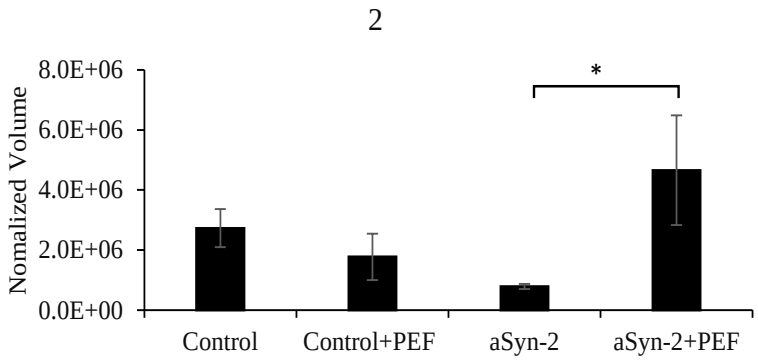
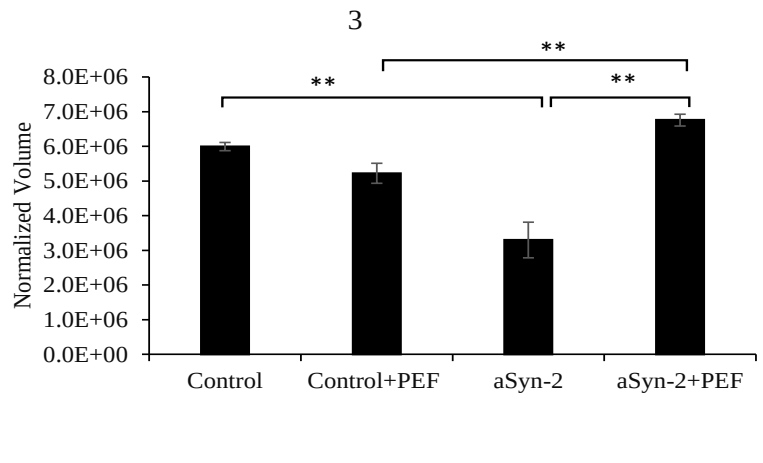
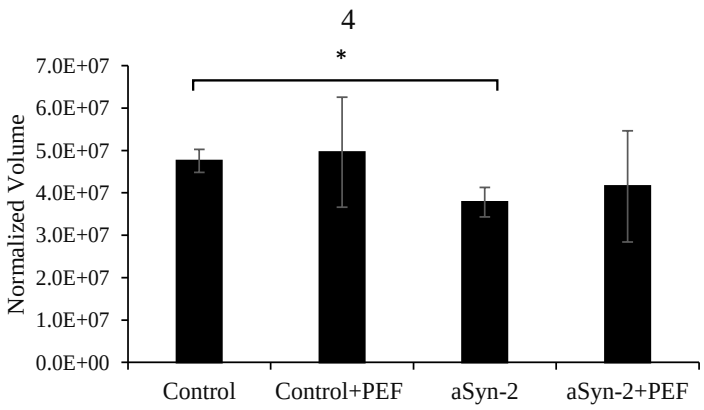
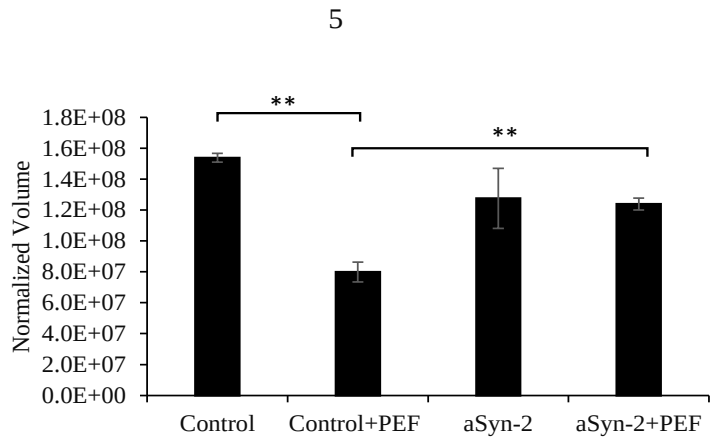
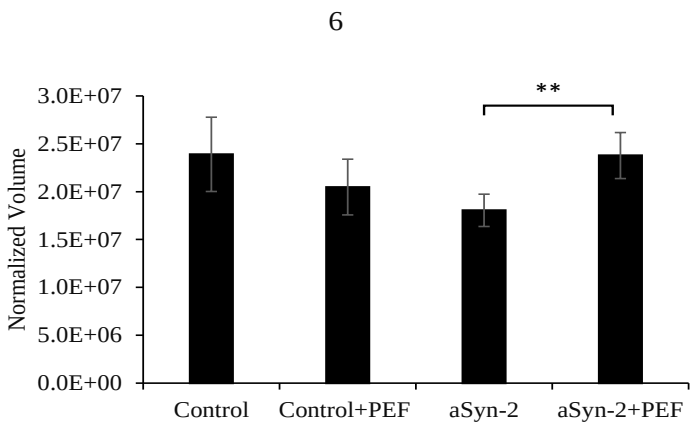
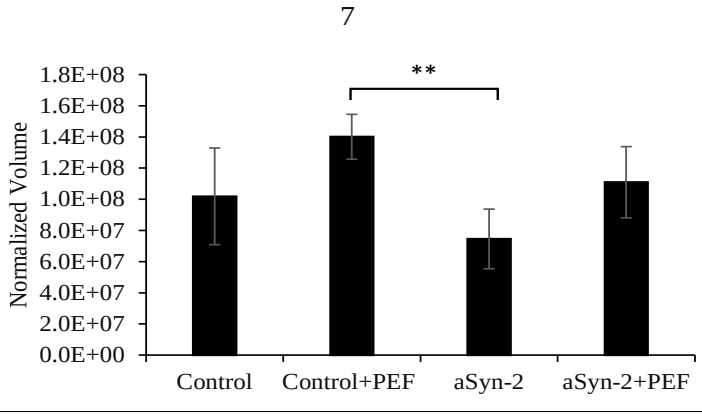


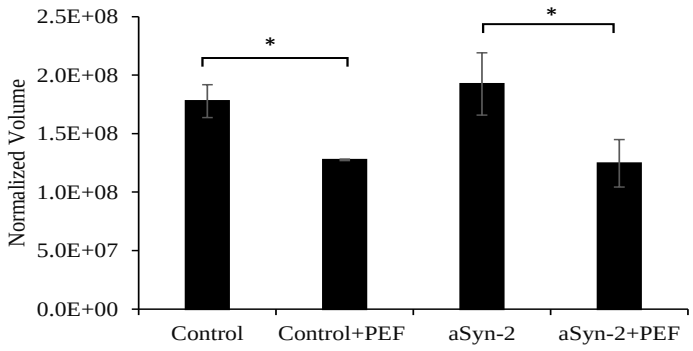
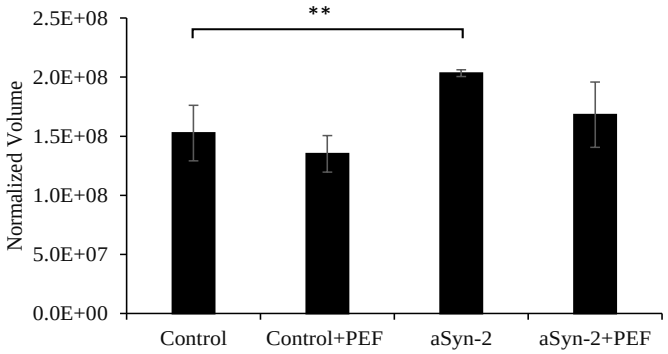
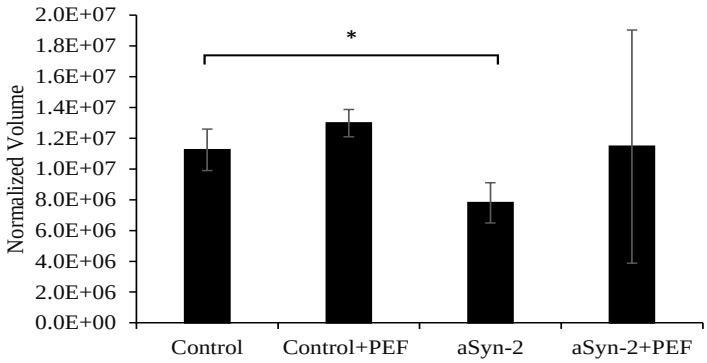
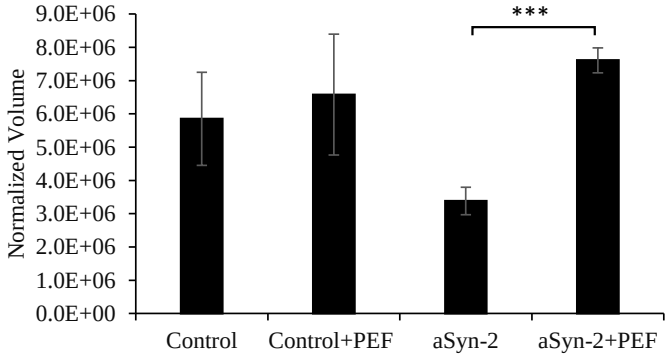
Figure 13: Representative 2-DGE gel of control strain cell proteome. (75 µg) The gel was CBB stained. The normalized volumes were compared to the controls (control strain) in order to estimate variations on protein content. In total 22 spots showing statistical significant differences among the four conditions in the study (control; control+PEF; aSyn-2; aSyn-2+PEF). Spots that were differentially abundant with statistical significance in all replicates were identified by Progenesis SameSpots image analysis. Further numbered spots with differential expression pattern (table 3) will be sent for protein identification by MS. IEF was performed with 3-10 NL IPG strips.Three independent biologic replicates were done for each condition.

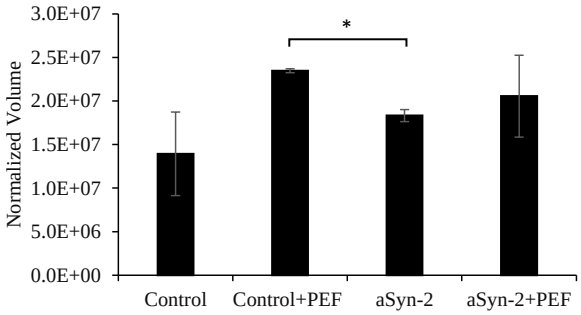
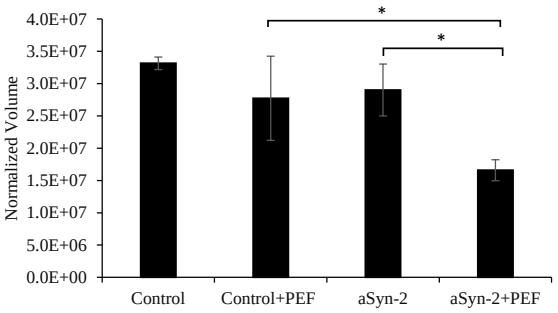
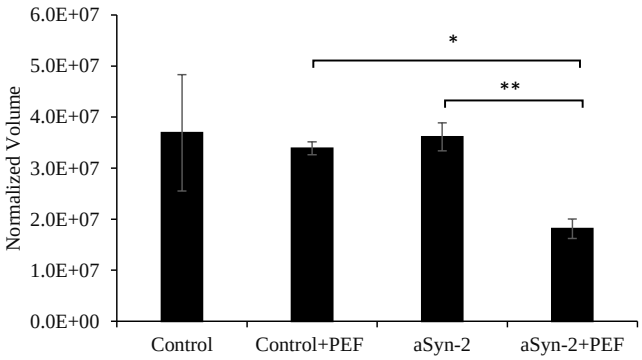
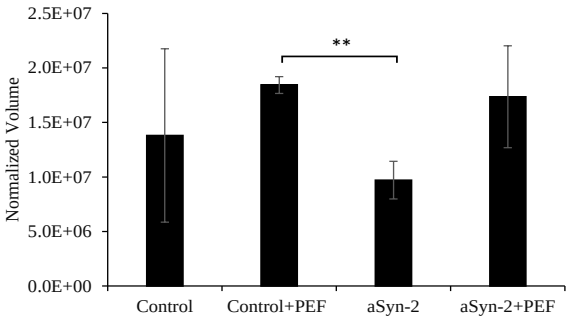
Through statistical analyses of the proteomes for all treatments it was possible to identify a total of 22 spots with different expression levels. The normalized volume for each protein spot differentially expressed among conditions and some characteristics of proteins such as, pI and MW, are described in table 3.

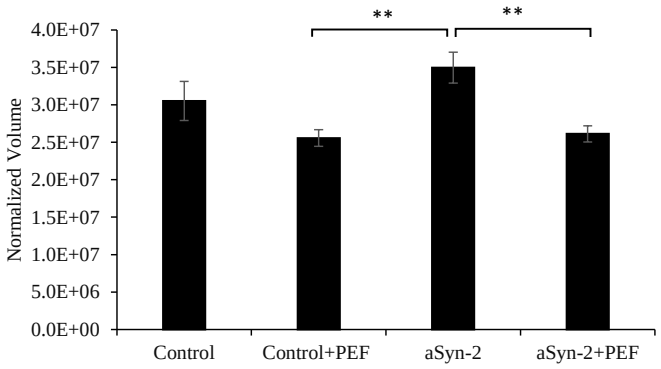
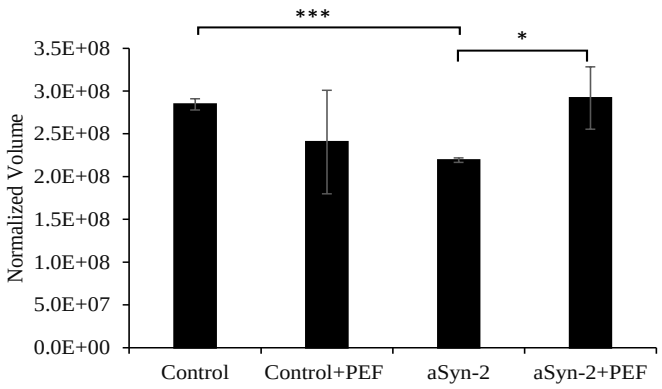
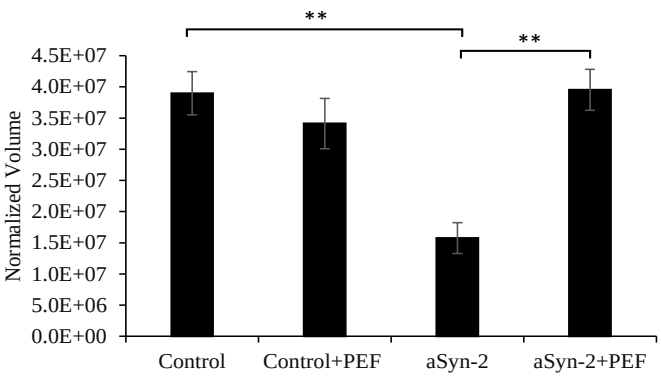
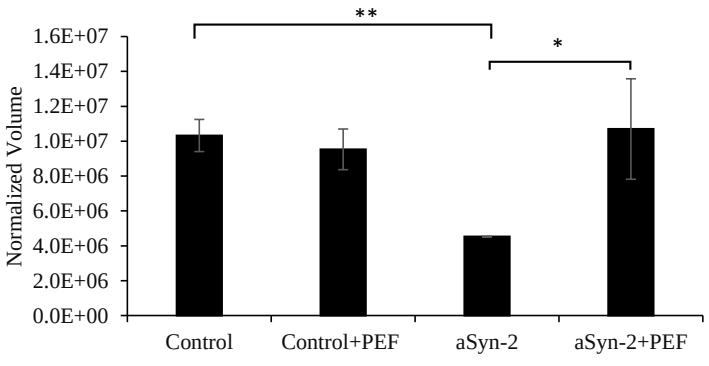
Table 3: Normalized volumes for protein spots differentially expressed among the four conditions in the study: control; control+PEF; aSyn-2; aSyn-2+PEF, and the respective pI and MW (kDa). Differences between treatments are denoted as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

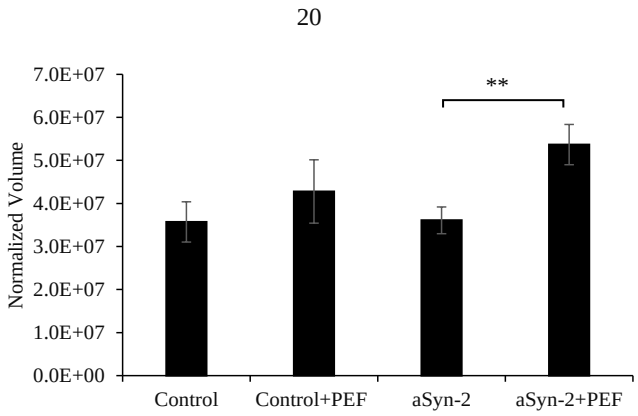
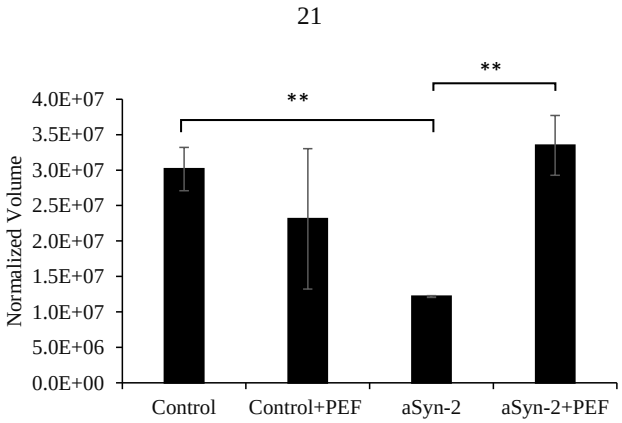
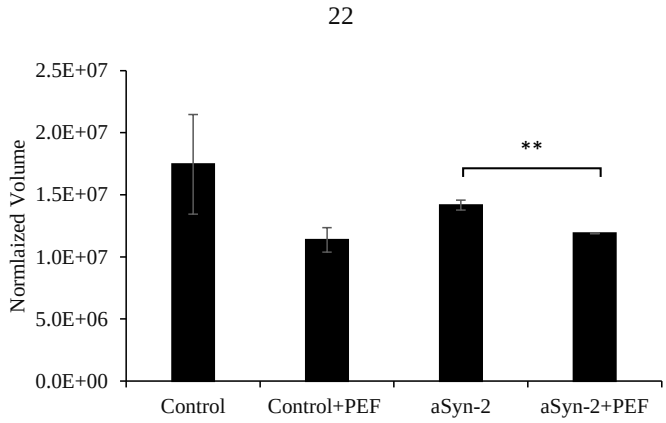
Spots number and normalized volumes	MW (kDa) i	pI ii
	27.2	5.3
	43.3	6.1
	53.2	6.7

	46.4	7.2
	31.7	6.1
	27.9	5.6
	36	7.9

8 	31.7	6.2
9 	55.9	6
10 	52.9	5.5
11 	51.6	6.6

12 	57.3	5.8
13 	25.8	8.9
14 	25.7	8.8
15 	40.8	7.8

16 	52	6.1
17 	31.5	5.8
18 	52.3	8.4
19 	52.6	8

	52.6	6.1
	52.6	7.2
	28	5.6

i) Predicted MW (kDa) was obtained by using an ExPASy tool (http://expasy.org).
ii) Predicted pI was obtained by using an ExPASy tool (http://expasy.org).

Given the data in Table 3, the 22 identified spots show significant variations in protein content between various conditions. It was observed that in each spot can exist more than one condition to vary either upregulated or downregulated. Thus we grouped quantitatively the total number of spots presenting variation in a given condition under study (table 4).

Conditions	Nr. of spots with significance variation between conditions	Upregulated	Downregulated
control vs. control+PEF	2	(0%)	2 (100%)
control vs. aSyn-2	8	1 (10%)	7 (90%)
control+PEF vs. aSyn-2+PEF	5	3 (60%)	2 (40%)
aSyn-2 vs. aSyn-2+PEF	15	10 (75%)	5 (25%)

Table 4: Number of spots with expression variations in the respective conditions under study: control; control+PEF; aSyn-2; aSyn-2+PEF.

Interestingly, minor variations in protein expression appears between control and control+PEF. For instance the spot number 5 presented a downregulation on protein expression when treated with PEFs, whose expression increases in the presence of aSyn. However PEFs not change the protein expression in the presence of aSyn (table 3). On the other hand the spot number 8 present a reduction on protein expression when treated with PEFs, and this decrease keeps even in the presence of aSyn protein, indicating that *C. album* leaf PEFs are counter the effects of expression of aSyn protein (table 3).

Looking at the conditions control vs. aSyn-2 it was found that remarkably 90% of spots are downregulated by aSyn expression when compared to the control strain, indicating a modulatory effect on the proteome expression by aSyn protein. One example of that is the spot 18 were it was observed that aSyn expression affect the normal expression of the protein, and the treatment with *C. album* leaf PEFs recovery the normal expression of protein spot 18 (table 3). Another example is the spot 19, where it was seen the same behavioural, aSyn reduced the protein expression of the spot, and PEFs recovery the normal expression. Only the spot number 9 present higher expression in the presence of aSyn (table 3). Between the conditions control+PEF vs. aSyn-2+PEF, it was observed that spot number 1, 3 and 5 presented higher protein expression in aSyn-2+PEF condition comparatively to control+PEF. In contrast spots number 13 and 14 displayed lower expression level relatively to control+PEF (table 3).

Remarkably, the majority of protein spots (15/22) show significant variations at the level of expression between the conditions aSyn-2 and aSyn-2+PEF (table 4). This result suggests that most of the proteins change their expression level due to the presence of aSyn protein or in response to the presence of the *C. album* leaves PEF. 75% of these fifteen spots presents higher protein expression level in the condition aSyn+PEF relatively to aSyn condition (table 4). For instance in the spots 2, 3, 6, 11, 17, 18, 19 and 21 it was observed a much higher protein expression when cells expressing aSyn are pre-

treated with PEFs. This may indicate that the PEFs are allowing the recovery of protein expression affected by aSyn expression. On the other hand 25% of spots treated with PEFs are downregulated in relationship to aSyn condition. Examples of that are the spots 8, 13, 14, 16 and 22 where it was observed that the treatment with PEFs reduce the protein expression when compared to aSyn condition (table 3).

Altogether, treatment with the *C. album* leaves PEF appears to restore the normal level of expression in all the 15 spots, either by increasing or decreasing the expression level. This result is very interesting because both aSyn protein and *C. album* leaves PEF appears to modulate the expression of the same protein, but in opposite ways, which indicates that possibly the PEFs may be acting in signalling pathways similar to aSyn. In fact, polyphenols can activate signalling pathways underlying neuronal survival, for example they are capable of inducing neurogenesis trough the activation of PI3 kinase-Akt[59,63]. Proteasome impairment is one of the characteristics of the abnormal deposition of misfolded proteins, like aSyn and some studies show an increase in the clearance of these misfolded proteins due to an increase in proteasome activity, promoted by polyphenols[53]. This may also be a possible mechanism of action of *C. album* leaves PEF.

Despite these promising results we have to wait for the results of mass spectrometry, in order to realize what proteins and subsequent signaling pathways are being affected for aSyn expression and C. album leaves PEFs. The proteins whose relative abundance was consider to vary in response to aSyn expression or C. album leaves PEF treatment will be sent for protein identification by MS.

- **SDS-PAGE-detection of phosphoproteins**

In order to study the presence phosphorylation activity in control and aSyn-2 strains, and find possible clues about the effect of polyphenols in our model yeast strains, we performed a SPS-PAGE and subsequently stain the gel with Pro-Q DPS (section Pro-Q® Diamond in material and methods). Yeast cells were grown as described in section Media and growth conditions. After protein extraction and quantification, 10 µg of protein samples of all the conditions (control; control+PEF; aSyn-2; aSyn-2+PEF) were subjected to an SDS-PAGE (section SDS-PAGE in material and methods). The results are show in figure 14.

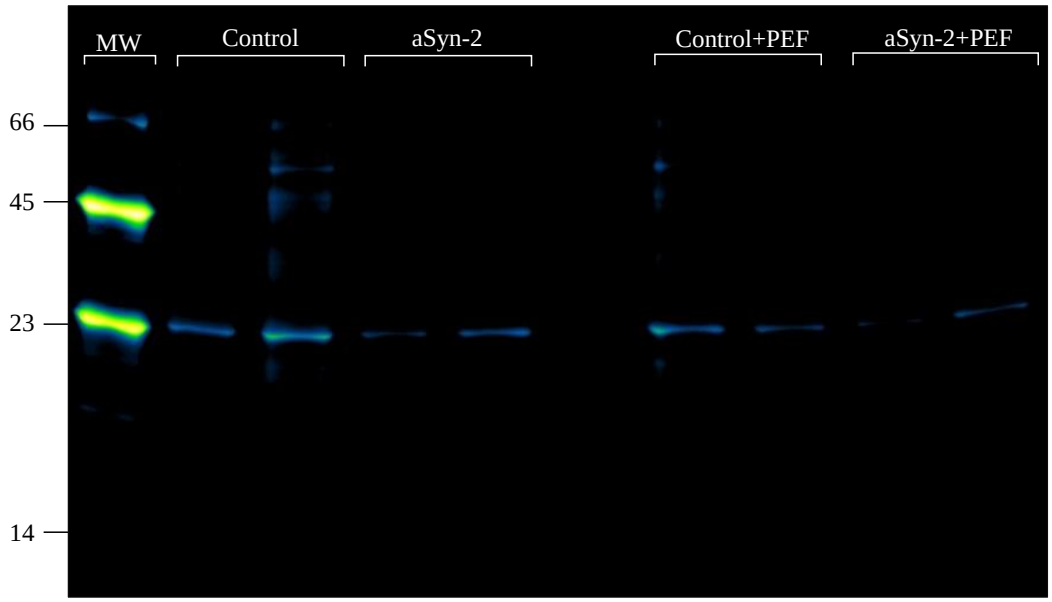


Figure 14: Protein phosphorylation profile of yeast strains incubated with *C. album* leaves PEF (control+PEF; aSyn-2+PEF) and without treatment (control; aSyn-2). 10 µg of protein sample of each condition was applied. SDS-PAGE gel was stained with Pro-Q DPS. Images of the gels were scanned in a laser imager with 532-nm excitation and 580 bandpass emission filter FLA-5100 Fuji Photo Film Co, Ltd. A representative image is show with two independent biologic replicates for each condition.

Considering the results, it was observed that polypeptides which exhibit phosphorylation activity among the four conditions are found in the 23 kDa region. Slight differences for this polypeptides in terms of phosphorylation intensity were detected between yeast cells treated with *C. album* leaves PEF, respectively, control+Ca and aSyn-2+Ca and yeast cells without treatment (Fig 14). This result may indicate that *C. album* leaves polyphenols can modulate the phosphorylation activity in control and aSyn-2 yeast cells. Forward, we will try to relate and understand this result by studying the phosphoproteome of yeast strains.

- **Phosphoproteome of control and aSyn-2 yeast strains with *C. album* Leaf PEFs**

Knowing that the most abundant modification of aSyn in LBs is the phosphorylation of serine 129, it becomes pertinent to study the phosphorylation network of the proteome of our Parkinson yeast model, to seek for modulations of phosphorylation mediated by *C. album* leaves PEF. Thus, 2-D gels of control; control+PEF; aSyn-2 and aSyn-2+PEF were stained with Pro-Q DPS (section Pro-Q® Diamond in material and

methods). The utilization of Pro-Q DPS allowed the detection and quantification of phosphoproteins in all the conditions tested. The results are shown in figure 15.

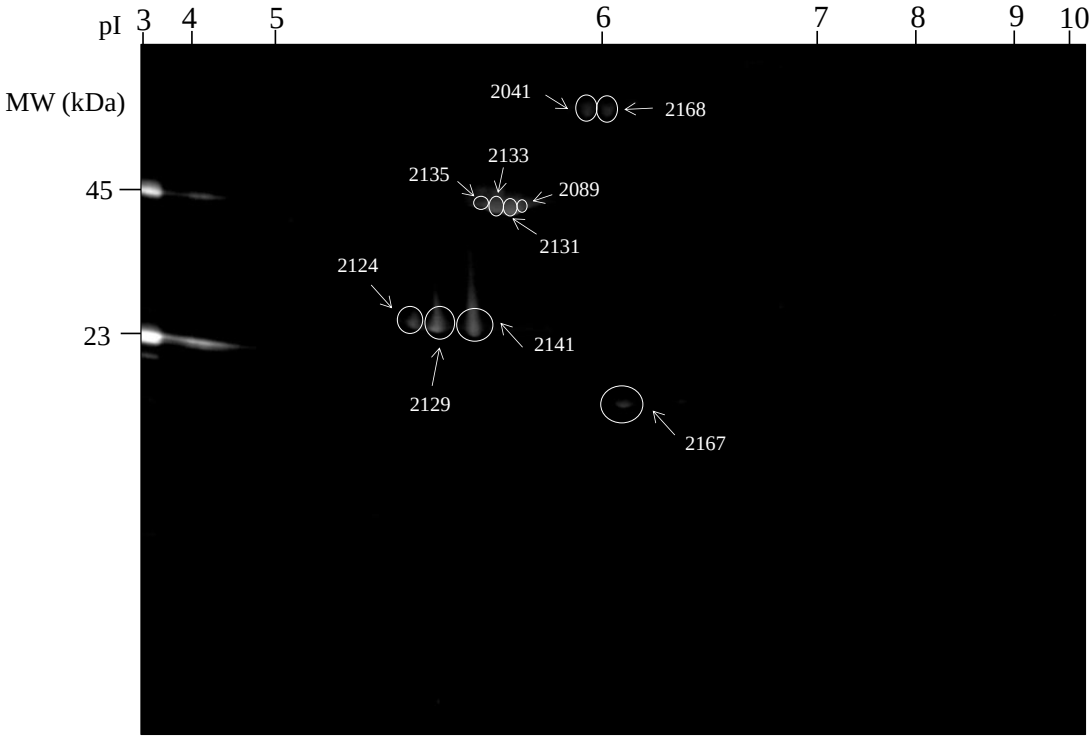
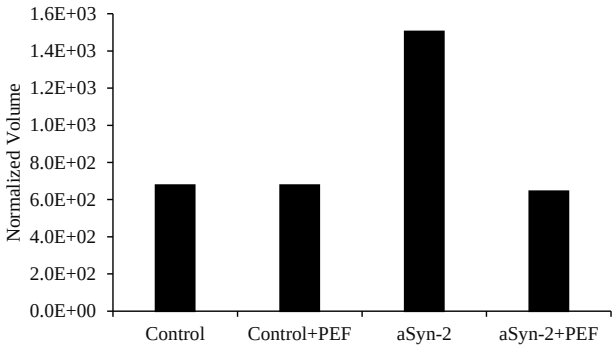
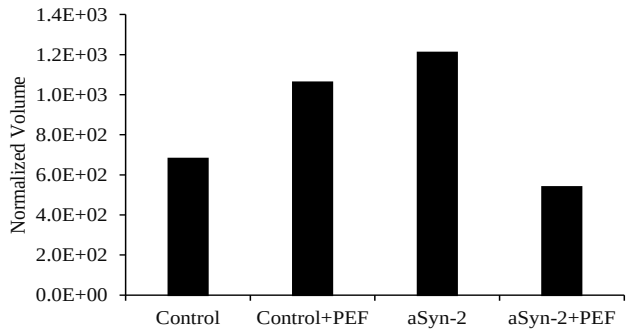
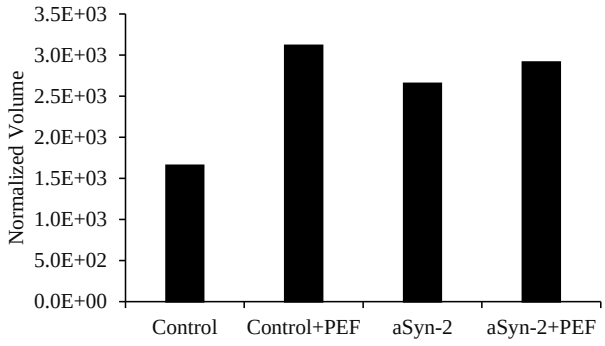
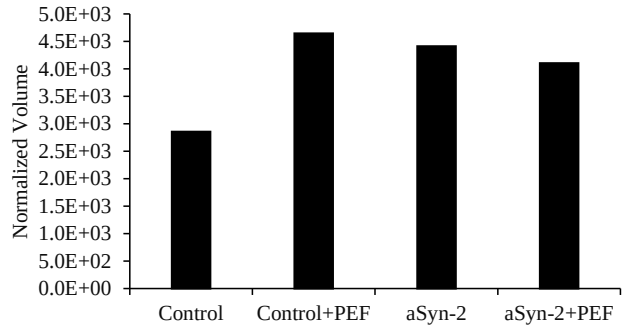
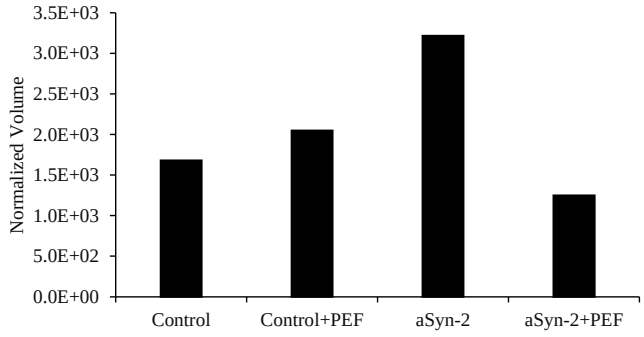
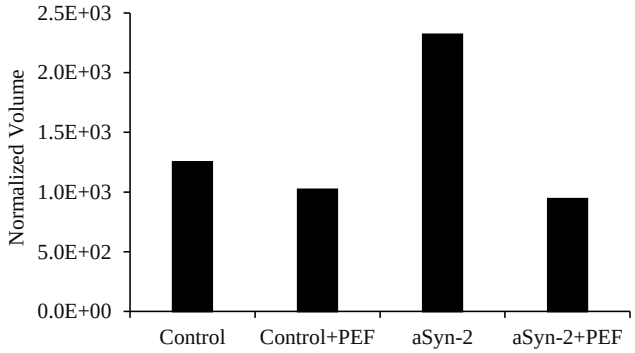
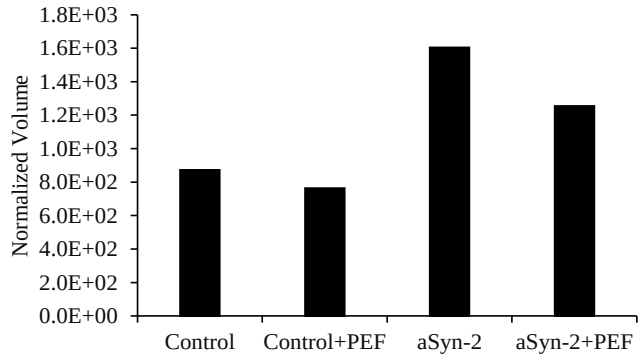


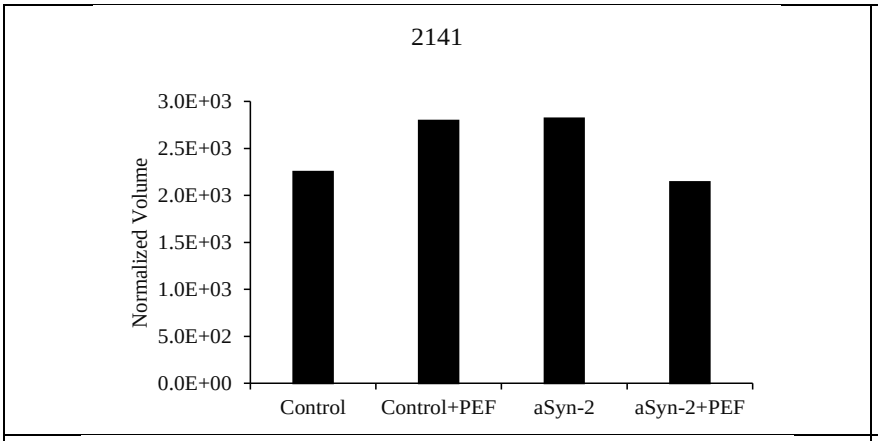
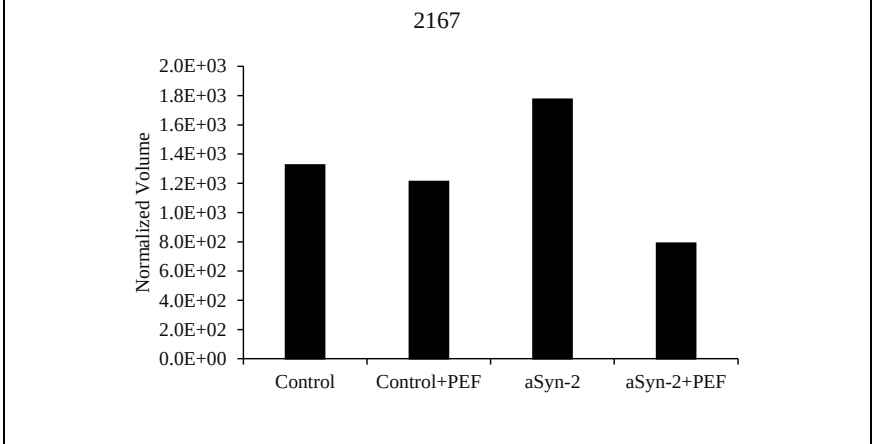
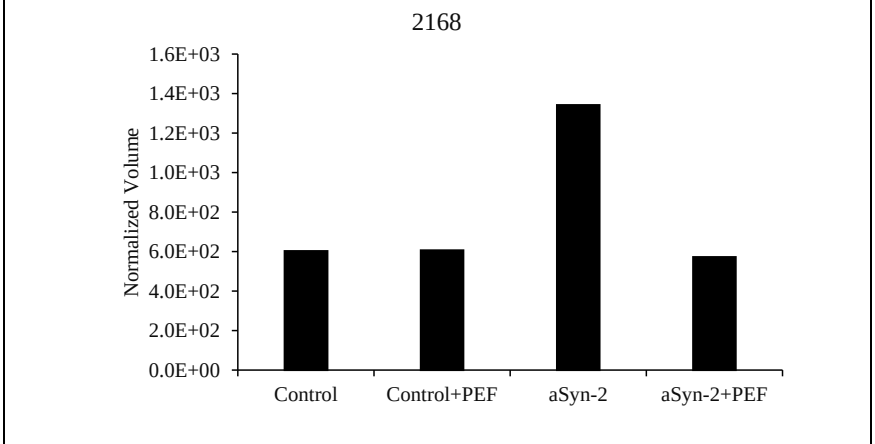
Figure 15: Representative 2-DGE gel of empty strain phosphoproteome (75 µg). The gel was stained with Pro Q-DPS. The normalize volumes were compared to the controls in order to estimate variations in phosphorylation levels. In total 10 spots exhibited phosphorylation signal between the four conditions in the study (control; control+PEF; aSyn-2; aSyn-2+PEF). IEF was performed with 3-10 NL IPG strips.

Preliminary results of the phospoproteomes analysis of all treatments allowed to detect a total of 10 protein spots with variance in phosphorylation signal. The normalized volume for each protein spot differentially expressed among conditions and some of the characteristics of proteins such as pI and MW are described in table 5.

Table 5: Normalized volumes for protein spots presenting phosphorylation levels among the four conditions in study: contro; control+PEF; aSyn-2; aSyn-2+PEF, and the respective pI and MW (Kda).

Spots number and normalized volumes	MW (kDa) i	pI ii
2041 	64.5	6
2089 	44.7	5.8
2124 	28.9	5.4

2129 	28.9	5.5
2131 	44.7	5.7
2133 	47.1	5.8
2135 	47.9	5.6

	28.4	5.6
	21.9	6.1
	64.5	6.1

i) Predicted MW (kDa) was obtained by using an ExPASy tool (http://expasy.org).
ii) Predicted pI was obtained by using an ExPASy tool (http://expasy.org).

Considering the data of the table 5, and analysing the levels of phosphorylation exhibited by each protein spot in the respective condition, a trend was observed over the 10 spots. In fact, the majority of protein spots (8/10) of the condition aSyn+PEF displayed a lower phosphorylation intensity with respect to the proteins spots of aSyn condition. The modulation in the intensity of phosphorylation activity of the proteins presented by yeast cells that were incubated with *C. album* leaves PEF, may reflect some type of

modulation in phosphorylation pathways by *C. album* leaves PEF. Also, the aSyn expression increases the levels of phosphorylation in almost all protein spots (9/10) in comparison to control strain and control+PEF. Since there are several kinases involved in aSyn phosphorylation, especially casein kinases 2 (CK2) and 1 (CK1), and some studies related an inhibition of the activity of this kinases, mediated by polyphenols[86,87], possibly C. album leaf PEFs are interfering with this pathways. Maybe PEFs are acting as "competitive inhibitors" of phospho-donor substrate ATP, preventing the binding of kinase to receptor, and stopping the respective signalling cascade[28,86,87]. This may be a mechanism that lead to decreased phosphorylation in cells treated *C. album* leaves PEF.

- **C. album leaves PEF effects on aSyn protein**

In order to assess how treatment with polyphenols affect the behaviour/expression of aSyn protein, a western blot was performed. Control and aSyn-2 yeast cells were growth as cited in section (Media and growth conditions) material and methods, supplement with 30 µg of *C. album* leaf PEF. Then 10 µg of total protein was submitted to SDS-PAGE. After the electrotransfer of polypeptides, immunodetection was performed as describe in section (Western Blot and Immunodetection) material and methods. The results are show in figure 16.

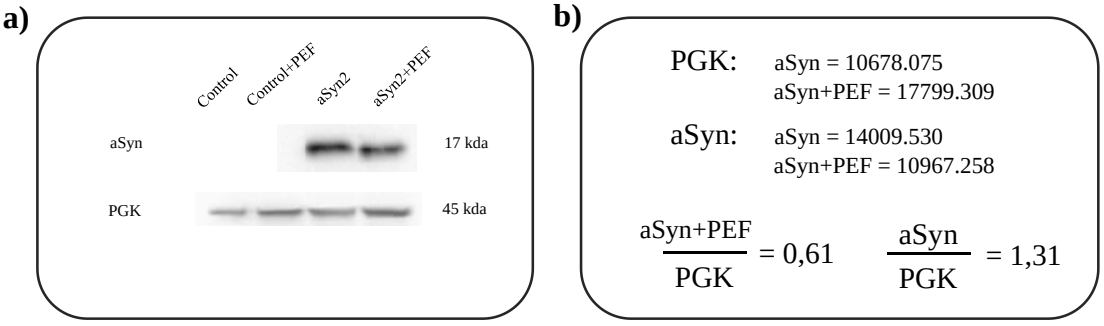


Figure 16: aSyn expression profile treated with *C. album* leaf PEF. aSyn expression levels in yeast cells assessed by western blot analysis of total protein extract. **a)** aSyn expression levels of control and aSyn-2 yeast cells subjected to the treatment with *C. album* leaf PEFs. **b)** Relative densitometric intensities. PGK was used as an internal loading control. Protein siganls were detected using the chemiluminescence detection kit FemtoMax Super Sensitive Chemiluminescent HRP Substracte. A representative image is shown. 3 independent biological replicates were performed.

When yeast cells expressing aSyn protein were treated with *C. album* leaf PEF it was observed a reduction in the content of aSyn protein. The relative densitometric intensities (Fig.16b) demonstrated that 30 µg *C. album* PEFs effectively reduced the level of aSyn protein in aSyn-2 yeast cells. Moreover and based on the previous results of

phosphoprotein analysis, is clearly very interesting to evaluate aSyn phosphorylation attenuation due to PEFs. This can be done by western blot targeting several known phosphorylation sites on aSyn protein, using specific antibodies. For instance S129, Y39, Y125, and Y133. Considering results of altered expression of aSyn and previous results from DSB lab, where PEFs incubation reduce the percentage of cells with aSyn inclusions, both in yeast model and H4 neuroglioma cells. Moreover, *C. album* leaf PEF increased the degradation of aSyn by autophagy in yeast and H4 cells[46]. These observations, suggest that protection promoted by *C. album* leaf PEF may be related with increased degradation of aSyn by autophagy.

aSyn can be degraded either by the UPS or by autophagy pathway. Interestingly, overexpression of aSyn causes impairment in proteasome system, thus autophagy pathway is required to compensate the increased protein accumulation and potential damage. Autophagy is a key degradation route for a wide range of intracytoplasmic aggregate proteins, and is considered as a pro-survival mechanism to protect cells from cellular stress or poor nutrient conditions[88]. Indeed recent evidence suggest that autophagy is mostly a cytoprotective mechanism that allow cells to recycle damaged organelles[89,90].

Several polyphenols have been associated with an increase in autophagy pathway, for instance resveratrol and curcumin[88–90]. This regulation in autophagy pathway by polyphenols it is related to the activation of Sirtuins, especially SIRT-1. Sirtuins are a class III histone deacetylases and have been show to regulate various physiopathological processes, including autophagy pathway. Once activated, SIRT-1 can deacetylate essential autophagic modulators such as ATG5 and ATG7, promoting autophagy. Furthermore, SIRT-1 deficient mice have accumulation of damages organelles and disruption of homeostasis[89,91,92] (Fig.17). Another way to increase autophagy in cells is related to downregulation of mammalian "Target of Rapamycin" (mTOR), an evolutionarily conserved protein kinase for modulating autophagy. Curcumin efficiently silence mTOR enchaning autophagy and resveratrol suppressed mTOR signalling in a S1RT-1-dependent manner[88–91]. Taken all these clues into consideration, it is reasonable to ponder that *C. album* leaf PEFs can enhance autophagy in yeast cells expressing aSyn, in a similar way to resveratrol for instance, leading to a decrease in aSyn inclusions,

ameliorating the cell fitness (Fig.17). Overall modulation of autophagic pathways by polyphenols could be a novel neuroprotective strategy for PD.

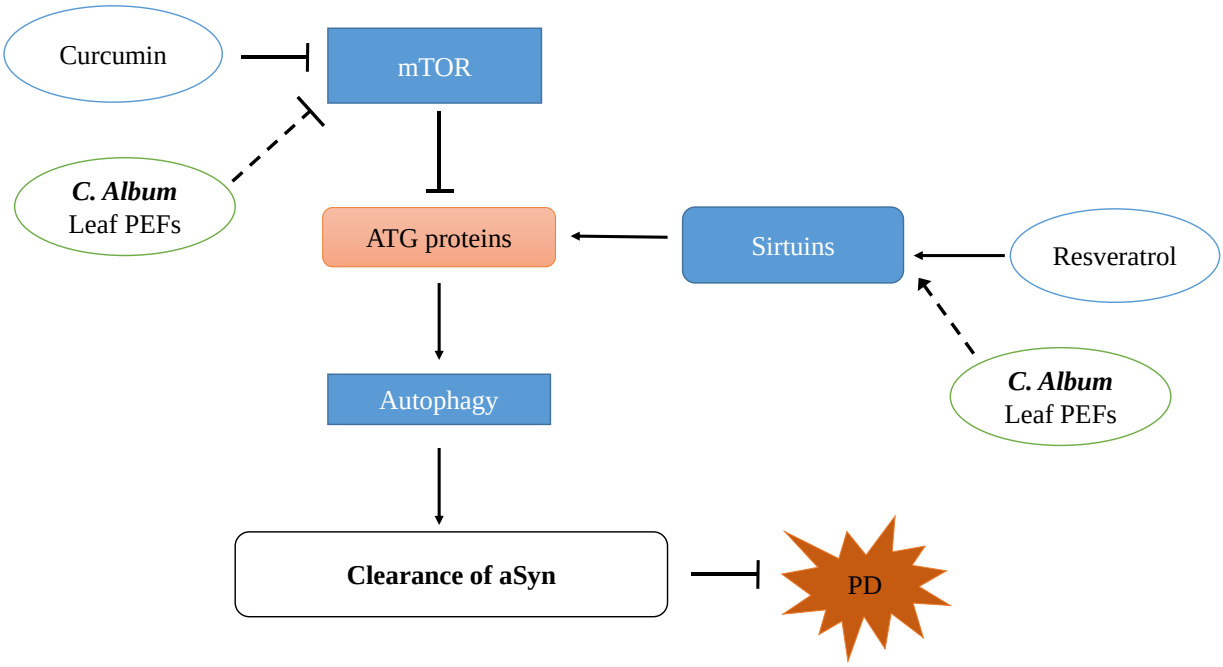


Figure 17: Schematic model of the role of Curcumin and Resveratrol in autophagy and possible modulation in autophagy mediated by *C. album* leaf PEFs. Resveratrol causes activation of Sirtuins, more precisely SIRT-1, followed by the induction of ATG proteins, essentials for the autophagic machinery. This pathway may be affected in the same way due to C .album leaf PEFs (dashes line). Also mTOR is downregulated by curcumin, and potentialy could be downregulated by C. album Leaf PEFs (dashes line). Induced autophagy enhances the clearance of aSyn, thereby may contribute to the neuroprotection on PD.

Based on this information's, in further analysis complementary to phosphoproteomics, it's possible to target this signalling intermediaries. For instance the induction of autophagy by *C. album* leaf PEFs can be determined by detecting protein levels of the autophagosomal membrane form of microtubule-associated protein 1 light chain 3 (LC3) using immunoblotting assay with anti-LC3 antibody[90]. Also we can access the expression level of ATG5 and ATG7, which are directly modulated by SIRT-1activity[88]. Another possibility, is the use of transmission electron microscopy in order to detect authophagosome structures of authopagy in cell lines treated with *C. album* leaf PEFs[89,90].

Final consideration and future perspectives.

Neurodegenerative diseases are among the most complex and puzzling of human disorders. Currently there is no effective treatments for these devastating illness, and are thus a social and economic burden for modern society, and one that will certainly get worse in the future due to the ageing of population. Among neurodegenerative disease, PD is one of the most prevalent worldwide. PD is a chronic progressive neurodegenerative disorder characterized by aSyn inclusions known as Lewy bodies, which are associated with progressive loss of dopamine neurons in SNpc. The selective neurodegeneration featuring PD has been linked to various intracellular processes particularly, mitochondrial dysfunction and subsequent oxidative stress. As well conformational changes of proteins affecting the UPS. All this with the aggregation of aSyn protein in the spotlight.

Since, current PD medications treat symptoms, none halt or retard dopaminergic neuron degeneration, there is an urgent demand for the identification of novel therapeutic agents that are capable of counteracting the progression of PD.

Polyphenols are the most abundant pytochemiclas in the human diet, and appear as possible candidates for protective agents in PD. The consumption of polyphenol-rich foods, especially fruits and vegetables, translates into benefits for human health. Polyphenols besides to be free radical scavengers, are now considered to modulate cellular mechanism of action, such as modulation of signalling pathway related to cell survival. *C. album* a Portuguese native species were chose for this study in order to evaluate the protective effects against aSyn protein, mediated by PEFs from *C. album* leaves. *S. cerevisiae* was used as a eukaryotic cell model to study the effects of aSyn expression and the bioactivity of PEFs.

Thus in this work we focus on the changes caused by aSyn protein on the proteome and phosphoproteome of yeast cells expressing two copies of aSyn protein, and also on the alterations in the same proteomes mediated by treatment of yeast cells with *C. album* leaf PEFs. With this analysis it is possible to study the protein network of our PD yeast model, and modulations mediated by PEFs, that will allow understanding the influence of these protective compounds in molecular mechanisms with this disease in particularly. Yeast growth parameters analysis revealed that aSyn expression is toxic and drastically decrease the growth of yeast cells comparatively to the control strain. Remarkably, it was demonstrated in this work that treatment with *C. album* leaf PEFs reduce aSyn induced

toxicity and enhances the viability of yeast cells. Our data from the growth curves and phenotypic assays validate that *C. album* leaf PEF is a promising protective agent against aSyn induced toxicity.

Through the proteomic tools used in this work was possible to study the proteome and phosphoproteome of our yeast cells in order to find changes in protein expression mediated by aSyn protein expression and *C. album* leaf PEFs. The analysis of the total proteome by 2DGE revealed 22 proteins spots that presented variations in their expression pattern among the conditions studied. Interestingly, it was found that 90% of spots are downregulated by aSyn expression when compared to the control strain, indicating that the expression of aSyn protein is affecting normal expression of these proteins. Even more fascinating is the fact that treatment of aSyn-2 yeast cells with *C. album* leaf PEFs reverse the effects of aSyn on protein expression, allowing to restore protein expression to similar levels presented by the control strain.

Thereby becomes evident that *C. album* polyphenols exert their neuroprotective actions, likely by the modulation of intracellular signalling cascades which control neuronal survival, death and differentiation[57,93,94].

In terms of phosphoproteome, with the preliminary results analysis it was possibly to detect 10 proteins that showed significant changes phosphorylation level. Remarkably the phosphoproteomes from yeast cells incubated with polyphenols from *C. album* leaf PEFs (aSyn-2+PEF) displayed reduced phosphorylation levels comparatively to yeast cells not treated (aSyn-2). Interestingly proteins of aSyn-2 strain which were not treated with polyphenols exhibit a higher level of phosphorylation. This preliminary findings suggest that *C. album* leaf PEFs are able to reduce the phosphorylation activity in our PD yeast model and counteract the increased phosphorylation levels mediated by aSyn expression.

This effect mediated by PEFs may be related to their ability of modulating signalling pathways linked to phosphorylation. Further studies using *C. album* leaf PEFs and targeting single phosphorylation sites in aSyn protein can provide information regarding the interactions of PEFs with specific sites of phosphorylation.

Moreover, upon incubation with *C. album* leaf PEFs there is a reduction in the level of aSyn protein present in yeast cells expressing the protein. Given this result and previous observations from DSB lab indicating that PEFs reduces the percentage of yeast cells with aSyn inclusions, suggest that the protection promoted by C. album leaf PEFs may be related with increased degradation of aSyn protein by autophagy[46].

Although the results obtained from proteomic approaches, various enhancements can be made to obtain more accurate and reliable results. For instance, could be interesting to fractionate our sample, in order to reduce the complexity of protein/peptide mixtures and separate various groups of proteins for subsequent analysis. Also, detect phosphorylation species directly from complex sample mixtures it's really difficult since phosphorylation often occurs at low stoichiometry. Thus enrichment of phosphoprotein and phosphopeptides techniques can be implemented[71,74,76]. Furthermore, the development of Phos-tag™ techniques offers a new method to detect phosphorylation activity[95]. This method provides a phosphate affinity SDS-PAGE for mobility shit detection of phosphorylated proteins. All these methodologies/optimizations can be used in further studies in order to complete and improve the results obtained in this work.

Intense efforts are being made to unravel the mysteries underlying neurodegenerative diseases. However, there still much to be learnt and discovered. Further technological advances are still required to lead to final goal of reaching 100% protein coverage in proteome studies, allowing characterization of all protein species in a given organism. This is of extreme importance to better understand the biologic mechanisms involved in PD and other neurodegenerative diseases, and more importantly, to discover novel agents for therapeutic interventions.

Budding yeast has been successfully used to perform studies in this work, it was demonstrated that polyphenols from *C. album* leaves can improve the viability of yeast cells expressing aSyn protein, and thus, protect yeast cells from aSyn induced toxicity. Thereby elucidating the beneficial health properties and neuroprotective potential of plant polyphenols.

Annex 1 - Western Blot - aSyn Immunodetection

With the purpose of verifying the expression of aSyn protein by strain aSyn-2 with different galactose concentrations in the culture SC medium, immunodetection in membrane was performed (section Western blot and immunodetection). Control and aSyn-2 yeast cells were growth as mentioned in section Media and growth conditions, and shifted to SC liquid medium supplemented with various galactose concentrations (0 %; 0,5 %; 1 % and 2 %). After growth, protein extraction and subsequent quantification was done using Lowry's method (section Protein quantification in material and methods). Samples with 10 μg of total protein was submitted to SDS-PAGE, and after the electrotransfer, immunodetection was performed using antibodies anti alpha-synuclein to detect expression of aSyn protein (section Immunodetection) (Fig.18).

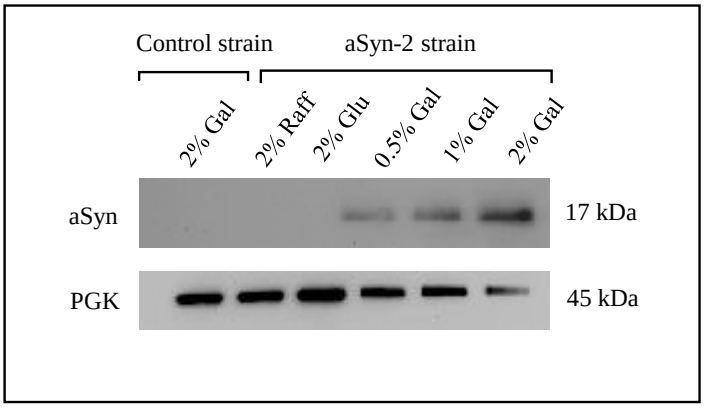


Figure 18: aSyn expression profile. aSyn expression levels in yeast cells assessed by western blot analysis of total protein extract. Different concentrations of galactose were used to optimize the better condition to express high levels of aSyn. PGK was used as an internal loading control. Protein siganls were detected using the chemiluminescence detection kit FemtoMax Super Sensitive Chemiluminescent HRP Substracte. A representative image is shown.

It was found that aSyn expression in aSyn-2 strain yeast cells only occurs on media containing galactose, as expected. It was also observed that the expression of the protein increases with the increase of galactose concentration present in the medium (Fig. 18). As predictable, control strain don't show any expression of aSyn protein. These results confirms that galactose at a concentration of 2% is the optimal condition to induce aSyn expression.

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